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SKIMMED MILK AS A SUPPLEMENT TO CORN IN FEEDING.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Alfred J. Wakeman.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, July 30, 1920.)

Practical experience has shown that corn-meal and skimmed milk form a combination which promotes the rapid growth of young animals as well as poultry. Laboratory experience has lately shown that the good results obtained with this mixture are due to the fact that the nutritive deficiences of the corn-meal are made good by the constituents of the milk. As the high cost of these food products makes it important to use them in the most efficient proportions, we undertook the experiments described in this paper. These were made with the following considerations in mind.

The food intake of animals is chiefly determined by their calorific requirements. In agricultural practice corn or corn-meal usually furnishes the major part of the calories of the ration. If skimmed milk is fed separately and before the corn-meal the animal may readily eat more than enough to supplement properly the corn, if fed milk after the corn it may eat too little. The proper way to feed these products, therefore, would be to mix them so that both may be eaten at the same time. Such a method would be particularly advantageous for those who have an irregular supply of skimmed milk because in this way all the latter would be used under conditions of maximum efficiency. Furthermore each individual animal would get more nearly its proper quota of the

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

milk than if it were allowed to drink it from a trough in company with others.

Our first experiment was designed to show how efficient a mixture of equal weights of skimmed milk and corn-meal would be and what deficiencies, if any, it might have. We intended to repeat this experiment using a smaller proportion of skimmed milk if the mixture of equal parts proved adequate, but this has been rendered unnecessary by experiments recently described by Hart and Steenbock[1] who state that

"... a highly efficient protein mixture is not obtained until the proportion of liquid milk to corn-meal reaches 1:1; in this proportion the milk nitrogen will constitute approximately 30 per cent of the total nitrogen of the ration.

These data relate only to protein efficiency and of course are not to be interpreted as indicating the amount of milk necessary for providing an adequate supply of vitamines or salts to an animal with a growth rate similar to that of swine."

Since their experiments supplement ours and ours supplement theirs in respect to the features indicated we here present our results with the approval of Hart and Steenbock.[2]

We fed four albino rats on a mixture of 91.5 per cent of yellow corn-meal and 8.5 per cent of "Krystalak," a preparation of dried skimmed milk containing 35 per cent of protein (N × 6.38) and 2 per cent of fat. The skimmed milk thus furnished one-quarter of the 11.55 per cent of protein which the food contained, while the milk fat was equal to only 0.16 per cent of the food mixture. The Krystalak contained 8.37 per cent of ash, thus contributing to the food mineral salts equal to only 0.7 per cent of the mixture.

Hart and Steenbock's experiments show that the protein in this food should be adequate, both in quality and quantity. According to Steenbock and Boutwell,[3] 88 per cent of yellow corn-meal supplies enough of the fat-soluble vitamine for normal growth and reproduction of albino rats when a salt mixture con-

[1] Hart, E. B., and Steenbock, H., *J. Biol. Chem.*, 1920, xlii, 167.

[2] As these authors have stated that they have complete experiments with rats similar to those they have reported with pigs, we did not feel at liberty to anticipate their publication without consulting them.

[3] Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 81.

taining sodium chloride, calcium lactate, and ferric citrate is also added, and the corn proteins are supplemented with casein. We should therefore expect our food mixture to be adequate in respect to this food accessory. Since 71 per cent, or more, of cornmeal has been shown by McCollum, Simmonds, and Pitz[4] to sup-

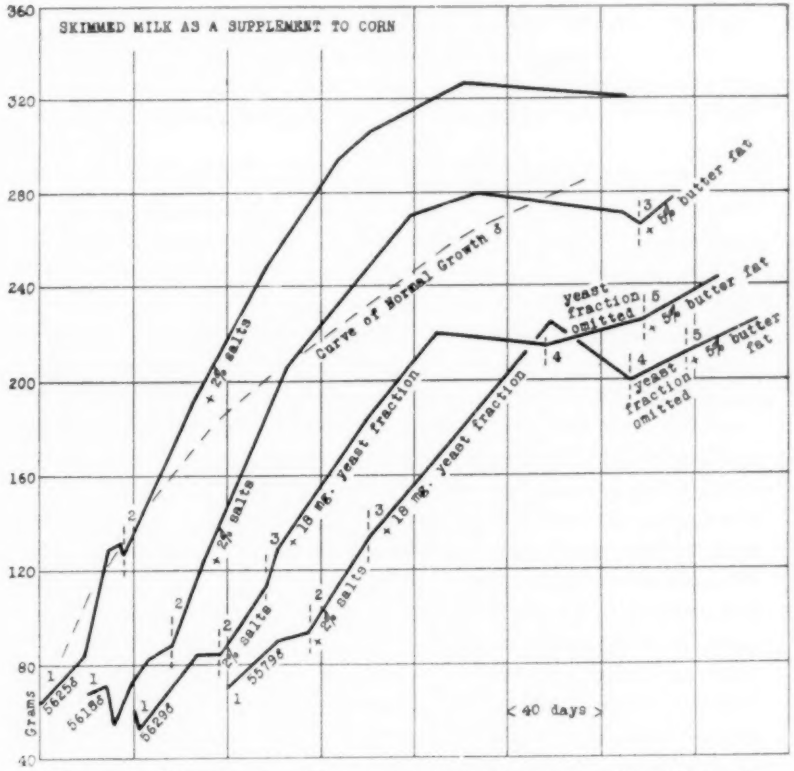

CHART 1. Showing the effect of supplementing corn with milk in the food of growing rats. In Period 1 growth was limited by the lack of sufficient suitable salts in the mixture. When these were supplied in Period 2 and subsequently by addition of 2 per cent of our salt mixture, good growth was secured. Addition of a vitamine preparation obtained from yeast (Rats 5629, 5579) did not exhibit any advantage, thus suggesting that skimmed milk and corn in the proportions used furnish sufficient water-soluble vitamine. Other details are referred to in the text.

[4] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxviii, 153.

ply enough of the water-soluble vitamine for the normal growth of albino rats, there is no reason to suspect that our food mixture is deficient in this respect.

The only question that deserves serious consideration is whether or not the salts supplied by the milk sufficed to supplement adequately the known mineral deficiencies of the corn. In the proportions used the 8 per cent of Krystalak contributed only 0.7 per cent of inorganic matter to the ration. The results of our experiments show that this is not enough, as it was necessary to add some of our standard salt mixture[5] before normal growth was made.

Chart 1 shows that Rat 5625 grew at a rate greater than the normal without any other addition to the diet than the salt mixture. Under the same conditions Rat 5618 grew somewhat better than at the normal rate for 160 days, but during the next 80 days it lost a little weight. This was recovered within 2 weeks when 5 per cent of butter fat was added to its ration. During a considerable part of the time Rats 5629 and 5579 received 18 mg. daily of a concentrated preparation of the water-soluble vitamine from yeast, an amount previously proved to supply alone enough of this vitamine for normal growth. The chart shows no beneficial effect after the addition of this preparation, or any unfavorable effect when it was discontinued. These rats did not grow so well as the other two, their rapid early gains during the first 130 days being followed by very slow growth. After 218 days of feeding, the addition of 5 per cent of butter fat caused no better growth.

These experiments indicate that this mixture of yellow corn-meal, skimmed milk solids, and salts may be slightly deficient in the fat-soluble vitamine. In practice such a mixture rarely forms the sole diet of young animals and when, as is generally the case, some green fodder is eaten this possible deficiency would be provided for. It is possible that the deficiency in mineral matter would also be compensated in this way, but it would be well to add about 1 pound each of calcium carbonate and sodium chloride[6] per 100 pounds of corn-meal to be on the safe side.

[5] The composition of this salt mixture is given by Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

[6] *Cf.* McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 165.

RESPIRATION OF CEREAL PLANTS AND GRAINS.*

II. RESPIRATION OF SPROUTED WHEAT.

By C. H. BAILEY and A. M. GURJAR.

(From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, St. Paul.)

(Received for publication, June 21, 1920.)

Respiration in frost-damaged wheat has been observed by us[1] to be at a higher level than in normal or sound wheat of the same moisture content. This was believed to be due, in part at least, to the higher percentage of dextrose and other simple carbohydrates in the frozen wheat. It then appeared desirable to determine the effect of sprouting upon the respiration of the grain, to ascertain whether or not this form of unsoundness in bulk grain could be expected to affect its keeping qualities.

A quantity of normal hard spring wheat was divided into three portions: one of which was wetted and allowed to sprout for 24 hours in the room at a temperature of $\pm 20°$; the second was sprouted under the same conditions for 48 hours; while the third was used in its original state as a control. The sprouted wheats were rapidly dried by spreading them out in a thin layer and blowing air over and through them with an electric fan. They were thus reduced to a moisture content of about 12 per cent. Each of the three samples was divided, and the several portions brought to different percentages of moisture by the addition of varying quantities of water. After standing for 3 days each of these was sealed in a tower, incubated at 37.8°C. for 4 days, and the respired CO_2 determined.

Chemical analysis of the several samples showed the normal wheat to contain 0.16 per cent of reducing sugars (calculated as

* Published with the approval of the Director as Paper 204, Journal Series, Minnesota Agricultural Experiment Station.

[1] Bailey, C. H., and Gurjar, A. M., Respiration of stored wheat, *J. Agric. Research*, 1918, xii, 685 (note pp. 697–699).

dextrose), while the samples germinated 24 and 48 hours contained 0.59 per cent and 1.11 per cent respectively, the ratios of the three samples being 14.4 : 53.2 : 100 in the order named.

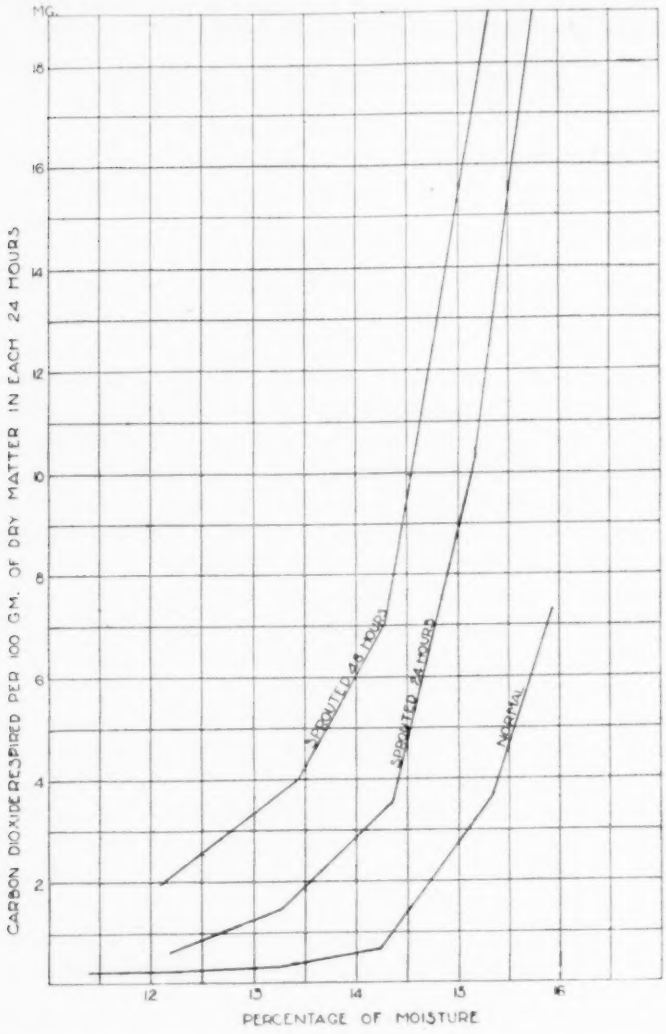

FIG. 1. Graph showing the relative rates of respiration of normal and sprouted wheat.

The quantity of CO_2 respired by each of these samples with different percentages of moisture is shown in Table I and in Fig 1. The latter indicates that at moisture contents of 12 to 16 per cent the sprouted grain respires decidedly more vigorously than the normal grain. At 12.5 to 14 per cent of moisture the ratios are approximately 1 : 4 : 10 for the normal, the 24 hour, and the 48 hour sprouted samples, which are not far different from the ratio of their reducing sugar content.

TABLE I.

Respiration of Normal and Sprouted Spring Wheat.

Normal.		Sprouted 24 hrs.		Sprouted 48 hrs.	
Moisture.	CO_2 respired per 100 gm. of dry matter in each 24 hrs.	Moisture.	CO_2 respired per 100 gm. of dry matter in each 24 hrs.	Moisture.	CO_2 respired per 100 gm. of dry matter in each 24 hrs.
per cent	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*
11.39	0.216	12.19	0.615	12.10	1.950
12.27	0.255	13.26	1.454	13.44	4.010
13.24	0.320	14.36	3.580	14.28	7.032
14.23	0.680	15.16	10.310	15.87	24.720
15.33	3.660	16.01	24.270		
15.94	7.320				

The shapes of the curves are somewhat different as well. Thus we find the acceleration of respiration with increasing moisture to be rather gradual between 12 and 14.5 per cent of moisture in the case of normal wheat, while the sprouted wheats show much greater acceleration between these limits.

These data establish that respiration, and consequently the quantity of heat energy released per unit of time and material, proceeds at a higher rate in sprouted grain than in normal wheat. This would be of significance in commercial storage of bulk cereals, and in grading grain when the grading system takes cognizance of respiration and keeping qualities in storage.

RESPIRATION OF CEREAL PLANTS AND GRAINS.*

III. RESPIRATION OF RICE PADDY AND MILLED RICE.

By C. H. BAILEY and A. M. GURJAR.

(From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, St. Paul.)

(Received for publication, June 21, 1920.)

In our paper on the respiration of stored wheat[1] we discussed the seat of respiration in the wheat kernel. It appeared, from published data, that respiration was most active in the germ or embryo, and lowest in the endosperm. To test this hypothesis further, we secured a series of samples representing rice paddy, and its milled products at different stages of milling. These were furnished by Mr. J. Mitchell Jenkins, Superintendent of the Rice Experiment Station at Crowley, Louisiana, who kindly obtained them from one of the Crowley rice mills. The rice was of the Blue Rose variety, 1,000 average kernels of which weighed 29.67 gm.

We proceeded to compare the respiration of the rough rice or paddy with that of the rice from the "stone" and from the "huller." That from the stone had practically all the hulls removed, as well as part of the germ, while the rice from the huller had advanced another stage in the milling process, and had most of the bran and germ removed. Portions of each of these three kinds of material were brought to different percentages of moisture by the addition of varying quantities of water. The treatment accorded them and the method of determining respiratory activity were similar to those employed in the wheat studies reported in the paper mentioned above.

In Tables I and II and Fig. 1 is shown the relative respiratory activity of these samples. Fig. 1 and Table II indicate plainly

* Published with the approval of the Director as Paper 205, Journal Series, Minnesota Agricultural Experiment Station.

[1] Bailey, C. H., and Gurjar, A. M., Respiration of stored wheat, *J. Agric. Research*, 1918, xii, 685.

that the rice paddy respires much more vigorously than the milled rice, and that, as the milling proceeds, the rate of respiration

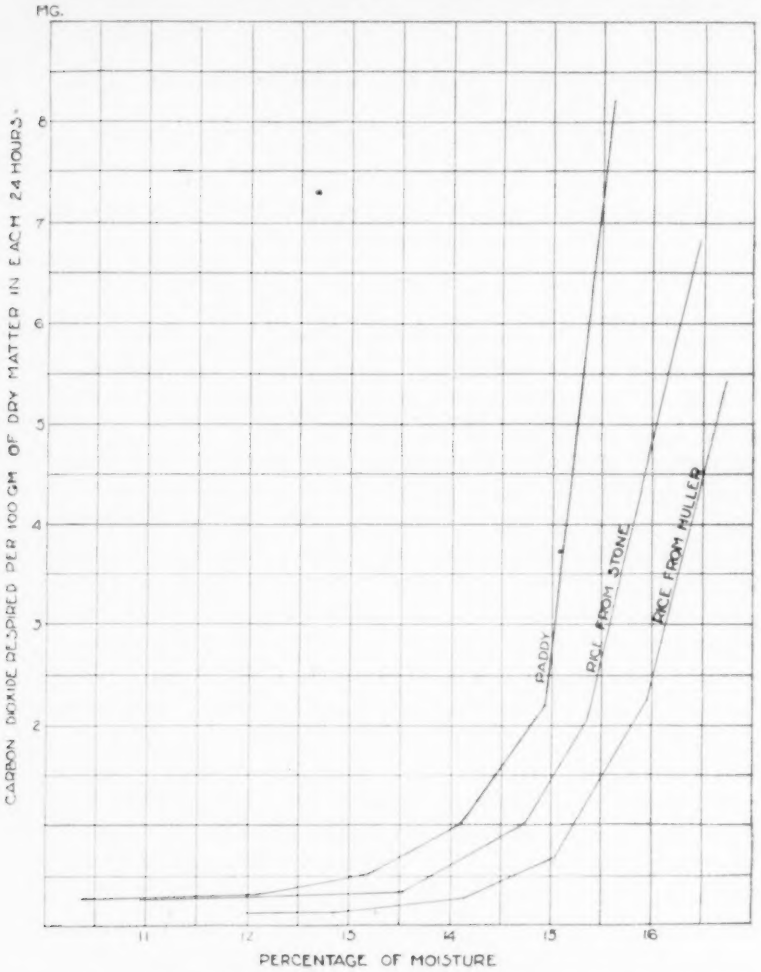

FIG. 1. Graph showing the relative rates of respiration of rice paddy, rice from stone, and rice from huller.

diminishes. Thus, the rice from the stones respired approximately 0.6 and that from the huller about 0.3 times as vigorously as the paddy.

Since the data presented in Table I were of necessity obtained in working with lots which did not vary uniformly in moisture content, the respiratory activity of each lot at even percentages of moisture was computed. These interpolated data are given in Table II, and establish the relative respiratory activity of the several lots more satisfactorily than the values given in Table I.

TABLE I.

Respiration of Paddy and Milled Rice Incubated at 37.8°C. for 4 Days.

Paddy.		Rice from stone.		Rice from huller.	
Moisture.	CO_2 respired per 24 hrs. for each 100 gm. of dry matter.	Moisture.	CO_2 respired per 24 hrs. for each 100 gm. of dry matter.	Moisture.	CO_2 respired per 24 hrs. for each 100 gm. of dry matter.
per cent	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*
10.37	0.27	10.93	0.27	11.99	0.13
12.15	0.32	13.54*	0.35	12.87	0.14
13.22	0.52	14.75	1.03	14.14	0.29
14.12	1.01	15.35	2.06	15.03	0.68
14.93	2.20	16.47	6.82	15.95	2.27
16.11	8.22			16.73	5.43

TABLE II.

Interpolated Quantity of Carbon Dioxide Respired per Unit of Time and Material at Even Percentages of Moisture.

Sample.	CO_2 respired per 24 hrs. for each 100 gm. of dry matter.				
	12 per cent moisture.	13 per cent moisture.	14 per cent moisture.	15 per cent moisture.	16 per cent moisture.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Paddy.....	0.32	0.48	0.95	2.56	7.65
From stones.....	0.29	0.32	0.60	1.46	4.70
" huller.....	0.13	0.15	0.27	0.67	2.44

The acceleration of respiration with increase in moisture content was then computed, using, as in the studies on wheat, the formula $\frac{K_m - K_{m-1}}{K_{m-1}}$ in which K_m represents the rate of respiration at a particular percentage of moisture, and K_{m-1} represents the rate of respiration of the same sample containing 1 per cent of moisture less than K_m. These data are given in Table III

and show that the acceleration due to increasing moisture is much the same in all three samples. This is also indicated by Fig. 1. The acceleration, within the limits studied, is materially greater than was found in our work on wheat.[2]

TABLE III.

Acceleration of Rate of Respiration of Rice Paddy and Milled Rice with Increasing Moisture $\frac{K_m - K_{m-1}}{K_{m-1}}$.

Sample.	Acceleration between the following percentages of moisture.			
	12 to 13 per cent.	13 to 14 per cent.	14 to 15 per cent.	15 to 16 per cent.
Paddy	0.50	0.97	1.69	1.99
From stones	0.10	0.88	1.43	2.22
" huller	0.15	0.80	1.48	2.64

The results of this study appear to support our previous conclusion that the germ or embryo is the seat of the most active respiration in a typical grain. It also shows that with different cereals the relative acceleration of respiration with an increase in the moisture content of the grain varies with different species, being greater in rice and its milled products than in hard wheat.

[2] Bailey and Gurjar,[1] p. 694.

RESPIRATION OF CEREAL PLANTS AND GRAINS.*

IV. THE RESPIRATION OF FROSTED WHEAT PLANTS.

By C. H. BAILEY and A. M. GURJAR.

(From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, St. Paul.)

(Received for publication, June 21, 1920.)

In a previous paper[1] we presented the results of a study of the respiration of wheat which had been frozen in the field before ripening. It appeared that the grain of such wheat, which had been thus frozen and subsequently thawed, respired more vigorously when stored in bulk than did normal wheat of the same moisture content. The greatest proportional difference was observed when the respective samples contained between 14.0 and 16.5 per cent of moisture.

To account for these differences between frosted and normal or sound wheat, it was postulated that, after freezing and then thawing, hydrolysis of certain complex carbohydrates probably proceeded at an increased rate. The products of this hydrolysis, and particularly the glucose thus produced, apparently constitute the substrate for the respiratory enzymes. The increased proportion of substrate may have accelerated the reaction induced by these enzymes and thus resulted in the respiration of more CO_2 per unit of time, and consequently the liberation of more heat energy.

It appeared that, if this hypothesis was sound, we should find that on freezing and then thawing the entire plant before it ripened a difference in the respiration of such material and of plants not frozen should be observed. We accordingly selected what appeared to be average plants in a field of Minnesota No.

* Published with the approval of the Director as Paper 206, Journal Series, Minnesota Agricultural Experiment Station.

[1] Bailey, C. H., and Gurjar, A. M., Respiration of stored wheat, *J. Agric. Research*, 1918, xii, 685.

169 bluestem wheat. Plants were cut at different stages of growth, the stems being severed 4 to 5 cm. above the ground level. This was done at 9.30 a.m. Half of these plants were immediately frozen by placing them in a refrigerating plant at a temperature of about −6°C. The following morning they were thawed in the air and then sealed in long glass tubes of about 5 cm. internal diameter. The other half of the plants, which were not frozen, were placed immediately after harvesting in similar glass tubes. Both lots were incubated in a dark thermostat for 24 hours at 30° and the respired CO_2 was determined by absorption in $Ba(OH)_2$ in the Truog tower as described by Gurjar.[2]

From the data thus secured it appeared that the frozen plants respired more vigorously during the first 24 hours after thawing than did plants which had not been frozen. The proportional

TABLE I.

Respiration of Normal and Frozen Wheat Plants.

Date cut.	Moisture in plant.	Weight per 1,000 kernels of dried grain.	CO_2 respired per 100 gm. of dry matter in 24 hrs.	
			Frozen plants.	Normal plants.
1917	*per cent*	*gm.*	*mg.*	*mg.*
Aug. 8	51.57	21.89	3,719	2,852
" 15	43.54	24.0	2,486	1,855
" 20	35.03	29.5	1,811	1,722

difference is not so great as was observed in the stored grain. It is possible, however, that the differences would have been greater had measurements been made after the material had stood a longer time after thawing.

The data given in Table I include the average percentage of moisture in the plants as cut, the weight per 1,000 kernels of the grain in the head after it had been cut and dried, and the CO_2 respired by the frozen and thawed, and the unfrozen plants. The grain was nearly ripe enough to harvest at the time the samples were taken on August 20, 1917. This is more or less evident from the weight per 1,000 kernels on that date, which is as great as the average weight of a similar number of ripe kernels.

[2] Gurjar, A. M., The adaptation of Truog's method for the determination of carbon dioxide to plant respiration studies, *Plant World*, 1917, xx, 288.

While no sweeping conclusions can be based on these data, they suggest that the extent of damage to the grain, as viewed from the milling and baking standpoint, might be indicated by the relative difference in respiration between plants frozen and thawed, and plants harvested at the same time but not frozen. As the plants approach maturity the relative difference between plants treated in these ways materially diminishes. The damage to the grain is generally conceded to diminish similarly. When a point is reached in the maturing of the plant where freezing and thawing do not accelerate respiration, it is highly probable that the grain will not be affected in its milling qualities by such treatment. That the moisture content plays an important part is indicated by the data. Desiccation accompanying maturity suppresses the activity of the enzymes responsible for respiration, and doubtless similarly suppresses the hydrolytic enzymes which unfavorably affect the quality of the grain.

RESPIRATION OF CEREAL PLANTS AND GRAINS.*

V. NOTE ON THE RESPIRATION OF WHEAT PLANTS INFECTED WITH STEM RUST.

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(Received for publication, June 21, 1920.)

During the crop season of 1917, a series of studies was conducted involving the respiration of wheat plants. In one phase of this work a number of rust-infected Marquis wheat plants were selected about 2 weeks before maturity, and cut a few centimeters above the crown of the plant. These plants were severely infected, being rated at between 80 and 90 per cent on the scale employed by the office of Cereal Investigations, United States Department of Agriculture.

The harvesting was done at 9.30 a.m., and the plants were sealed at once in glass tubes. At the same time a like number of plants of the same variety not infected with rust were cut and placed in similar tubes. Duplicates of both lots were secured, and these were placed at once in a dark thermostat at 30°C. After 24 hours the tubes were removed and the respired CO_2 was determined.

In Table I are shown the moisture content of the plants, weight of 1,000 kernels after drying, and milligrams of carbon dioxide respired in 24 hours (*a*) per 100 gm. of dry matter, and (*b*) per plant. The rust-infected plants had a decidedly lower moisture content than the sound plants on the day they were cut (August 9, 1917), due, presumably, to the accelerated transpiration of rust-infected tissues. The moisture content was actually as low as that of normal plants in an adjoining field a week later. The

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TABLE I.

Respiration of Normal and Rust-Infected Marquis Wheat Plants Harvested August 9, 1917.

Sample.	Moisture in plant.	Weight per 1,000 kernels of dried grain.	CO_2 respired in 24 hrs.	
			Per 100 gm. of dry matter.	Per plant.
	per cent	*gm.*	*mg.*	*mg.*
Normal plants	56.08	19.54	2,904	61.46
Rust-infected plants	46.41	20.44	1,608	39.16

kernels in the heads of the infected plants were somewhat heavier, although the difference is not significant. The rate of respiration of the infected plants was materially lower than the sound plants. If respiration of these tissues is in any sense a measure of metabolic processes it appears that metabolism was decidedly depressed in the rust-infected plants. This may afford an index to the metabolism of such infected material, and the consequent effect upon the filling of the kernel.

OCCURRENCE OF DIASTASE IN THE SWEET POTATO IN RELATION TO THE PREPARATION OF SWEET POTATO SYRUP.

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(Received for publication, July 18, 1920.)

The manufacture of syrup from sweet potatoes by the use of malt has long been a matter of public record.[1] Directions for the small scale production of sweet potato malt syrup were issued by the Department of Agriculture in February, 1919. The necessity for use of malt, however, apparently has prevented any wide use of the method.

Further work on the production of the syrup has unexpectedly revealed the fact that the sweet potato is so rich in diastase that nearly all the starch becomes converted into soluble carbohydrates by autolysis upon slow cooking. Moreover, the hot pulp formed by crushing the cooked sweet potatoes drains readily, thus permitting the easy recovery of the sweet juices. Sweet potato syrup, therefore, can easily be made without the use of malt.

The method consists simply in so heating the potatoes in the water that the tissues are heated at the temperature of maximum diastatic activity for from 10 to 20 minutes, then heating to boiling in order to soften the tissues, crushing, and separating the sweet juices from the insoluble pulp. The juice is then evaporated to syrup with or without further treatment.

For example, 1 kilo of Porto Rico potatoes was covered with water in an aluminum kettle, placed over a Fletcher burner, and the water heated to 60°C. The gas was then turned down and the heat applied very slowly so that the temperature gradually rose from 60 to 80°C. during an hour and from 80° to the boiling point during the next ½ hour. The boiling was continued for ½

[1] U. S. Patent 109,991 was granted to Charles Delamarre on December 6, 1870.

hour, when the potatoes were thoroughly soft. They were then mashed in the water in which they were cooked and enough water was added to form a thin pulp. The sweet juice in the pulp was then freed from the insoluble pulp ingredients by suction, using a Buchner funnel and filter paper, and the residue on the filter repeatedly exhausted with hot water. The filtrates were combined, evaporated to a thin syrup, and weighed. The weight was 714 gm. and the Brix reading at 20°C. was 37.6°. Thus, 714×37.6 or 268.5 gm. of syrup solids had been exhausted from 1 kilo of sweet potatoes. The dried pulp weighed 59 gm. The syrup was finally evaporated to a solids content of about 75 per cent. It was a slightly turbid, amber-colored liquid with a faint, pleasant odor and a sweet taste, with a slight flavor of the sweet potato. It contained a little soluble starch as shown by the iodine test, but not enough to cause it to thicken upon standing.

The sweet potatoes can be prepared for extraction by simply cooking them in water as in preparing for table use, but the resulting syrup will contain enough soluble starch to cause it to thicken upon cooling and standing. Tests on the diastatic power of extracts of sweet potato and sweet potato flour were made by Lintner's method as described by Brown.[2]

	Degrees Lintner
Porto Rico fresh pulp[3]	300
Nancy Hall " " [3]	125
Porto Rico flour[4]	500
Nancy Hall " [4]	300
Big Stem Jersey flour[4]	160

SUMMARY.

Sweet potatoes are high in diastatic power and it is possible to convert nearly all their starch into soluble carbohydrates by slowly cooking the potatoes in water. The pulp formed by mashing the cooked potatoes with hot water drains readily, permitting the easy recovery of the sweet juice.

[2] Brown, A. J., Laboratory studies for brewing students, New York, 1904.

[3] Reduced to pulp in a Herles press (Herles, F., *8th Internat. Congr. Appl. Chem.*, 1913, xxvi, 5).

[4] Produced by shredding sweet potatoes with beet knives, drying in a rapid current of hot air, grinding, and bolting.

CALCIUM REQUIREMENT OF MAINTENANCE IN MAN.*

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With the Cooperation of A. R. Rose and M. S. Rose.

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(Received for publication, July 29, 1920.)

The demonstration upon laboratory animals by Osborne and Mendel,[1] that calcium necessarily occupies a prominent place in the inorganic food supply, and that of McCollum, Simmonds, and Parsons,[2] that calcium is one of the limiting factors in a very large proportion of our staple foods, lends added interest to the measurement of the calcium requirement in man and the comparison of the amount of calcium required for normal human nutrition with the amounts found in the ordinary freely chosen food of representative families or other groups of people.

Results obtained in this laboratory several years ago[3] indicated that, in so far as could be judged from the limited data determined at that time, it was by no means safe to assume that a freely chosen food supply would always furnish calcium in quantity sufficient to afford a safe margin above the actual requirements of normal nutrition.

Further study, therefore, seemed desirable and the purpose of the present paper is to summarize the data of a considerable number of additional laboratory experiments and to compare them with the findings of further observations upon actual food supplies.

* Published as Contribution No. 342 from the Department of Chemistry, Columbia University.

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131.

[2] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 113, and in numerous other papers.

[3] Sherman, H. C., Mettler, A. J., and Sinclair, J. E., *U. S. Dept. Agric., Off. Exp. Stations, Bull. 227*, 1910

In addition to the experimental data published from this laboratory in 1910,[3] 1918,[4] and 1919,[5] of which the latter have been recorded without as yet being discussed, studies upon the calcium requirement of maintenance have been made upon three healthy men, Subjects I, E, and R.

Subject I, weighing 61 kilos, consumed bread, milk, wheat breakfast food, egg whites, apple (or banana), rice, butter, and coffee, his daily calcium intake during each of six successive 4 day experiments being 0.46, 0.41, 0.41, 0.42, 0.32, 0.32 gm., respectively. The corresponding output in each of the six experiments was: in urine, 0.15, 0.18, 0.20, 0.20, 0.20, 0.20; in feces, 0.42, 0.50, 0.26, 0.34, 0.32, 0.21 gm. per day, respectively (Experiments 10 to 15 in Table I).

Subject E, weighing 69 kilos (Experiments 56 to 58 in Table I), after a preliminary period on calcium-poor food, took during a 3 day experiment a basal ration of bread, butter, and fruit with a calcium intake of 0.18 gm. per day; the daily output being 0.42 gm. (urine, 0.34, feces, 0.08 gm.). Without intermission there followed another 3 day experiment in which milk was added to the diet bringing the daily calcium intake to 0.39 gm., the output being the same (urine, 0.28, feces, 0.11 gm.), so that equilibrium was established at this figure. Then meat (lean beef) was added to the diet with the result that the daily calcium intake was increased by 0.01 gm. and the output rose 0.16 gm. (urine, 0.36, feces, 0.19 gm.). This loss of body calcium following the addition of meat to the diet might have been thought to be due to the fact that the added meat gave to the diet as a whole an excess of acid-forming over base-forming mineral elements; but other data obtained in this laboratory (Sherman, Gillett, and Pope[4]) do not indicate any such marked relation between the preponderance of acid- or base-forming elements in the food and the amount of calcium needed for maintenance.

[4] Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383. Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 301.

[5] Sherman, H. C., Winters, J. C., and Phillips, V., *J. Biol. Chem.*, 1919, xxxix, 53.

TABLE I.*

Indicated Calcium Requirement for Maintenance per 70 Kilos of Body Weight per Day.

Experiment No.	Calcium.	Experiment No.	Calcium.	Experiment No.	Calcium.	Experiment No.	Calcium.
	gm.		*gm.*		*gm.*		*gm.*
1	0.29	25	0.64	49	0.39	73	0.50
2	0.28	26	0.44	50	0.61	74	0.60
3	0.82	27	0.56	51	0.43	75	0.50
4	0.42	28	0.70	52	0.58	76	0.41
5	0.35	29	0.54	53	0.53	77	0.37
6	0.66	30	0.53	54	0.53	78	0.42
7	0.46	31	0.39	55	0.58	79	0.40
8	0.66	32	0.49	56	0.30	80	0.40
9	0.61	33	0.55	57	0.29	81	0.39
10	0.65	34	0.48	58	0.40	82	0.42
11	0.78	35	0.35	59	0.28	83	0.39
12	0.54	36	0.28	60	0.28	84	0.33
13	0.61	37	0.30	61	0.27	85	0.45
14	0.61	38	0.27	62	0.29	86	0.33
15	0.49	39	0.32	63	0.30	87	0.50
16	0.40	40	0.34	64	0.60	88	0.37
17	0.42	41	0.29	65	0.55	89	0.54
18	0.44	42	0.46	66	0.59	90	0.40
19	0.33	43	0.42	67	0.54	91	0.31
20	0.41	44	0.41	68	0.55	92	0.44
21	0.35	45	0.43	69	0.47	93	0.35
22	0.40	46	0.46	70	0.46	94	0.29
23	0.40	47	0.50	71	0.40	95	0.33
24	0.53	48	0.47	72	0.55	96	0.38
						97	0.34
Average							0.45

* Experiments 1 and 2, Bertram, J., *Z. Biol.*, 1878, xiv, 354. No. 3, Renvall, G., *Skand. Arch. Physiol.*, 1904, xvi, 94. Nos. 4 and 5, von Wendt, G., *Skand. Arch. Physiol.*, 1905, xvii, 211. No. 6, Holsti, Ö., *Skand. Arch. Physiol.*, 1910, xxiii, 143. Nos. 7 to 9, Sherman, Mettler, and Sinclair.[3] Nos. 10 to 15, Rose, A. R., and Sherman, not previously published. Nos. 16 to 34, Sherman, Gillett, and Pope.[4] Nos. 35 to 48, Sherman, Wheeler, and Yates.[4] Nos. 49 to 55, Sherman, Gillett, and Pope.[4] Nos. 56 to 58, Sherman, not previously published. Nos. 59 to 63, Sherman and Osterberg, not previously published. Nos. 64 and 65, Sherman and Winters.[4] Nos. 66 to 75, Sherman, Winters, and Phillips.[5] Nos. 76 to 97, Rose, M. S., *J. Biol. Chem.*, 1920, xli, 349, and unpublished data.

Subject R, weighing 80 kilos, took during five consecutive 3 day experiments without intermission a diet of bread, butter, and apples furnishing an average daily intake for each of the five periods of 0.23, 0.21, 0.20, 0.21, 0.21 gm., respectively. The corresponding output for each experimental period was: in urine, 0.11, 0.17, 0.16, 0.18, 0.18; in feces, 0.21, 0.15, 0.15, 0.16, 0.16 gm. of calcium per day (Experiments 59 to 63 in Table I).

For purposes of comparison and discussion the data of all available experiments which seem to be quantitatively comparable (including, through the courtesy of Professor M. S. Rose, some unpublished experiments recently performed in her laboratory) have been computed to a uniform basis of daily calcium output per 70 kilos of body weight and the results brought together in Table I. Since calcium leaves the body so largely by way of the intestine, the error which may occur in the separation of the feces belonging to successive experimental periods is likely in the case of calcium to be more significant than in the case of phosphorus, and relatively much greater than in the case of nitrogen. Knowing by experience how readily a part of the calcium output properly belonging to an experimental period may thus be carried over into the one following, the writer has had this in mind in compiling the data of other investigators as well as those from his own laboratory and has sometimes treated two successive periods as one when convinced by examination of the protocols that a more trustworthy result is thus obtained. It will be seen that there are included in the table the data of 97 experiments (experimental periods of 3 to 8 days) showing an extreme range of 0.27 to 0.82 gm. and an average of 0.45 gm. per 70 kilos of body weight per day.

In the discussion of an analogous compilation of the data of experiments upon protein metabolism,[6] it was pointed out that in an appreciable proportion of those experiments the protein requirement was probably overestimated, largely because, influenced by previous overestimates of the amount of protein needed in nutrition, the experimenters did not reduce the protein intake to a low enough figure and for a long enough time really to determine the minimum amount on which equilibrium could have

[6] Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

been maintained. So far as this source of error is concerned the tendency in the study of calcium requirement has doubtless more often been in the other direction. In the past the calcium requirement of the body was underestimated. Experiments made to test calcium requirement have sometimes involved the use of diets furnishing so little calcium that the output (although greater than the intake) has been depressed below the point at which equilibrium could actually be maintained. Largely for this reason it is probable that some of the data for indicated calcium requirement in Table I, based as they are on the data of output when the intake was somewhat insufficient, are appreciably below the true requirements of the respective subjects. Doubtless there are other cases in which the requirement was overestimated through the use of diets unnecessarily rich in calcium for the purpose of the test; but these appear to be fewer in number and the general average of 0.45 gm. of calcium (equivalent to 0.63 gm. of CaO) is probably not above the true requirement.

Having now on record about 100 cases each of reasonably comparable experiments designed to measure the maintenance requirements for protein, phosphorus, and calcium, respectively, in normal human nutrition, it is of interest to compare the variability of the individual observations in the three series of experiments (each representing the work of several different investigators) and the probable errors of the three mean results as computed by accepted statistical methods.

The 109 experiments upon protein requirement[6] show a mean of 44.4 gm., a standard deviation of 9.07 gm., a coefficient of variation of 21, and a probable error of the mean of ± 0.58 gm.

In 95 experiments upon phosphorus requirement[7] the mean with its probable error is 0.88 ± 0.01 gm., the standard deviation is 0.15 gm., and the coefficient of variation is 17.

The 97 experiments upon calcium requirement tabulated above give a mean result of 0.45 gm. with a probable error of ± 0.008 gm. and show a standard deviation of 0.12 gm. and a coefficient of variation of 27.

In each of the three cases the data are now sufficiently numerous and consistent so that the probable error of the mean is

[7] **Sherman, H. C.,** *J. Biol. Chem.*, 1920, xli, 173.

less than two one-hundredths of its value and therefore presumably as dependable as the accepted statements of average composition of even the most familiar of our staple foods.

It will be noted also that the coefficient of variation has been found to be of the same order of magnitude in the experimental study of protein, of phosphorus, and of calcium requirements. If any significance is to be attached to the slightly higher coefficient of variation in the data for calcium over those for protein or for phosphorus, it must follow that the case of calcium is the one which would seem to call for the most liberal margin of intake over the estimated average maintenance requirement if individual variability is to be covered by an ample factor of safety. Apparently a large proportion of American dietaries are open to improvement in this respect.

Comparing the present average of 0.45 gm. per man per day as the indicated calcium requirement with the previously reported average of 44 gm. as an indicated protein requirement, and keeping in mind the fact that the figure for protein is more likely to be an overestimate than that for calcium, and that the relative probable error due to individual variations is very similar in the two cases, we are forced to conclude that a food supply, in order to furnish these essential nutrients in relative proportions corresponding to the needs of the body, should contain at least 1.0 gm. of calcium (or 1.4 gm. of CaO) for every 100 gm. of protein. In the large majority of American food supplies of families and larger groups, as indicated by dietary studies made before the war by the United States Department of Agriculture and the New York Association for Improving the Condition of the Poor, this has not been the case. It does not follow that the calcium content was necessarily too low in the majority of dietaries, but rather that the food supply furnished a much more liberal surplus of protein than of calcium. When the results of 224 presumably typical American dietary studies were calculated to the usual basis of nutrients per man per day the average protein content was found to be 106 gm., or 140 per cent above the indicated maintenance requirement of 44 gm., while the average calcium content was 0.74 gm., or 64 per cent above the estimated actual minimum of 0.45 gm. Probably more significant than the average content of all the dietaries is the proportion of cases falling

below the estimated requirement, either as regards protein, or as regards calcium. Only one of the 224 cases studied showed less protein than the indicated requirement, while one in every six was below the indicated requirement in calcium. In other words, the comparison of dietary studies or records of actual food supplies with the laboratory evidence of nutritive requirement suggests that very many more American dietaries are deficient in quantity of calcium than are deficient in quantity of protein, though of course there may be many cases in which the kind of protein, or the intake of some particular amino-acid, is unsatisfactory. If, in all the dietaries in the above group of 224 which showed less than 3,000 calories per man per day, the amount of food had been increased up to that level, there would have been no case of protein deficiency as judged by this standard while by the same criterion about 7 per cent or one in every fourteen of the dietaries studied would still be deficient in calcium. There seems to be no room for doubt that more attention should be given to the calcium intake both in human nutrition and that of farm animals. So far as the requirement for calcium in itself is concerned the intake may be supplemented by purely mineral additions as when the animal feeder includes finely ground rock phosphate ("floats"), bone ash, or oyster shells in the rations which he provides. Similarly calcium carbonate or phosphate might be habitually added to human food, either separately or by mixing it with the table salt used in seasoning; but it would probably be more difficult to persuade people generally to make such additions than to teach a more liberal use of foods naturally rich in calcium, while the latter course has the added advantage that the foods whose larger use would be invoked to increase the calcium intake (notably milk in its various forms) are important sources of proteins of high nutritive efficiency and of the fat-soluble vitamine as well.

THE PRODUCTION OF ACETALDEHYDE BY CERTAIN PENTOSE-FERMENTING BACTERIA.*

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The search for intermediate products in the fermentation of the carbohydrates has occupied the attention of scientists for many years. This search has been pushed most vigorously in connection with the alcoholic fermentation by yeast. The investigations of Neuberg, Harden, and Kostytschew are most conspicuous and have resulted in a better understanding of the biochemical changes that occur. Neuberg's theory (1) that pyruvic acid is an intermediate product in alcoholic fermentation is now generally accepted. This theory rests mainly on indirect evidence, although Fernbach and Schoen (2) and Beijerinck and Folpmers (3) reported the production and isolation of pyruvic acid from fermenting sugar cultures, and Mazé and Ruot (4) claim to have obtained it by the fermentation of lactic acid with bacteria. Neuberg found that pyruvic acid is fermented by yeast with the production of carbon dioxide and acetaldehyde and he isolated and identified the acetaldehyde thus formed. Although acetaldehyde has been observed many times in different types of fermentation, until recently no conclusive evidence has been obtained to show that it is an intermediate product in sugar fermentations. The occurrence in wine was noted by Magnes-Lahens (5) in 1855 and since that time its presence and the factors related to its occurrence have been the subject of numerous investigations (6). Trillat (7) and his associates as a result of their investigations concluded that acetaldehyde was not an intermediate product in the fermentation of sugar but arose from a secondary oxidation of the ethyl alcohol.

Buchner, Langheld, and Skraup (8) obtained small quantities of aldehyde in the fermentation of sugars with yeast juice and likewise concluded that it was derived from ethyl alcohol by oxidation.

Kostytschew (9) on the other hand regarded acetaldehyde as an intermediate product and by the addition of zinc chloride to a fermentation of

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glucose by yeast obtained enough aldehyde to prepare the *p*-nitrophenylhydrazone. The amount obtained was small and Kostytschew's conclusion that acetaldehyde is an intermediate product was vigorously assailed by Neuberg (10) and his coworkers.

In the fermentation of glucose by *Bacillus coli communis*, Grey (11) reported the production of a minute quantity of acetaldehyde. From the fermentation of 7.46 gm. of glucose he obtained 0.00234 gm. of acetaldehyde.

Numerous attempts have been made to increase the quantity of acetaldehyde by the addition of various substances. This was the object sought by Kostytschew with zinc chloride. In a later investigation Kostytschew (9) employed methylene blue to serve as an acceptor for hydrogen and thus to check the reduction of acetaldehyde to alcohol. The result was an increase in the yield of acetaldehyde. Later Palladin and Sabinin (12) found that methylene blue enhanced the production of acetaldehyde from lactic acid.

Müller-Thurgau and Osterwalder (13) increased the quantity of acetaldehyde formed in wine by adding small quantities of potassium *m*-bisulfite at varying intervals during the fermentation. Similar experiments with sucrose and glucose gave from 0.350 to 1.180 gm. of aldehyde per 100 gm. of sugar fermented. Previously Passerini (14) had obtained an increase in the aldehyde content of wines by the addition of bisulfite.

In an investigation of the factors that influence the formation and disappearance of acetaldehyde from wines Laborde (6) confirmed the observations of earlier investigators, that acetaldehyde may originate from the oxidation of alcohol or be formed as an intermediate product in the breaking down of sugar. His results showed a marked increase in the production of acetaldehyde when sulfurous acid was added to the fermenting solutions.

It was not until Neuberg and his coworkers (1, 15) employed a more suitable fixative that acetaldehyde was obtained in large amounts, and thus established the relation of acetaldehyde to the fermentation of sugar. With sodium sulfite as a fixative, he obtained acetaldehyde equivalent to 17.95 per cent of the sugar fermented. The aldehyde was determined by three different methods and was also isolated and identified by its hydrazone with *p*-nitrophenylhydrazine. The aldehyde-sulfite compound was isolated and identified as $(CH_3CHOH.SO_3Na)_2+H_2O$. These results present convincing and conclusive evidence for the existence of acetaldehyde as a product in the fermentation of sugars by yeast.

In a later publication, Neuberg and Nord (16) extended the work to the fermentation of sugars by bacteria and found that from 0.0014 to 0.2532 gm. of aldehyde formed in 100 cc. of culture containing 2 per cent of glucose. Indications of aldehyde formation by bacteria have been observed by others.

Harding and Ostenberg (17), in a study of the color changes produced by bacteria on the fuchsin-sulfite media of Endo, assumed that aldehyde is produced from the carbohydrate. At first the aldehyde combines with the sulfite and is thus prevented from oxidizing to acid, but later when the

sulfite is used up additional aldehyde undergoes oxidation to acid and in this way decolorizes the medium. They suggest that aldehyde formation may be used as a basis for the differentiation of colon and typhoid organisms.

De Bord (18) reported that in the breaking down of glucose and lactose by many kinds of bacteria, aldehyde is formed. The organisms were grown in tube cultures and aldehyde formation was tested with Schiff's fuchsin reagent. No evidence is presented to prove the identity of the aldehyde. Since Schiff's reagent is affected by many substances other than aldehyde, de Bord's results are by no means conclusive.

Buchner and Meisenheimer (19) suggested that in a fermentation yielding butyric acid, butyl alcohol, and other substances acetaldehyde is first produced. Harden (20), in a detailed study of the action of *Bacillus coli* and similar organisms on carbohydrates, advanced the theory that the carbohydrate is decomposed into an intermediate substance which, in turn, gives rise to formic acid and acetaldehyde. The formic acid later is converted into CO_2 and hydrogen.

The work described in this paper was planned primarily to study acetaldehyde formation by pentose-fermenting bacteria in the breaking down of both hexose and pentose sugars. Three widely separated types of bacteria were selected for this investigation. The first, to which has been given the laboratory number 26, was isolated from sunflower silage and apparently belongs to the colon-aerogenes group. Its biochemical and cultural characteristics will be described in a later paper. It ferments all the common sugars and in addition attacks vigorously the pentoses, xylose and arabinose. The second organism used was *Bacillus acetoethylicum*, isolated and described by Northrop and his associates (21). The third group of bacteria was the pentose fermenter *Lactobacillus pentoaceticus, n. sp.*, whose isolation and fermentation characteristics have been described in a previous publication (22).

EXPERIMENTAL.

In general the carbohydrates were fermented in a yeast water medium with and without peptone, to which an excess of calcium carbonate was added. In order to hold the aldehyde, the fixative calcium sulfite or sodium sulfite was used. The calcium sulfite was added to the yeast water before sterilization, and in the experiments where sodium sulfite was used a 20 per cent aqueous solution of the sulfite was sterilized and the desired amount of

this solution added to the culture. The calcium sulfite was freshly prepared from sodium sulfite and calcium chloride, air-dried, and used as the hydrated salt $CaSO_3.2\ H_2O$. The sodium sulfite contained a considerable amount of sodium sulfate and hence the figures given in the tables represent only approximately the concentration of sulfite in the medium.

The alcohol, volatile acid, and non-volatile acid were determined as described in previous publications. Acetaldehyde was determined according to the method developed by Neuberg and Nord (16). 100 cc. of culture were cooled with ice and an amount of barium chloride sufficient to precipitate the sulfite was added. After the addition of 10 gm. of calcium carbonate the flask was placed in an oil bath and joined to a long Allihn condenser. The delivery end of the condenser was connected to a thick walled Pyrex flask packed in a freezing mixture of ice and salt. A thistle tube partly filled with water was attached to the Pyrex flask to serve as an escape tube for the air in the apparatus. After about one-third of the contents of the flask had been driven over, steam was passed in and the distillation continued until about 400 cc. had collected in the receiving flask. The distillate was made up to a definite volume and the aldehyde in an aliquot determined by the iodine titration method (23).

Trial determinations with dry redistilled acetaldehyde (boiling point 20–21°C.) to which the above reagents had been added gave a recovery of from 98 to 100 per cent.

Acetaldehyde Production by Culture 26.

Culture 26 ferments the pentoses, xylose and arabinose, vigorously and tolerates a high degree of alkalinity. Since the culture solutions containing sodium sulfite are distinctly alkaline (pH 8.0) this organism is well adapted to the conditions existing in a sulfite medium. Three flasks of 2 per cent glucose-yeast water medium were inoculated with this organism and, after 24 hours of fermentation, 10, 20, and 30 cc. of a 20 per cent solution of sodium sulfite were added to the respective flasks. Here the concentration of the sulfite was 0.5, 1.0, and 1.5 per cent as calculated on the original volume. At frequent intervals the cultures were tested for aldehyde by means of sodium nitroprusside and diethylamine

(Simon's test as modified by Rimini (24)). The most satisfactory tests were obtained by mixing 5 cc. of culture and 0.5 cc. of a fresh 4 per cent solution of prusside, and carefully adding 5 to 10 drops of a 5 per cent solution of the amine so as to form a layer on top. After about 20 to 30 seconds, a sharp blue zone forms where the two liquids come together. If the test-tube is held against a white background, this zone becomes more distinct. In the presence of much aldehyde the blue color extends through the entire solution when the tube is shaken. No aldehyde could be detected until the 3rd day, when a slight test was found in Flask 3. After 5 days all the cultures gave a distinct blue color with these reagents. The intensity of color increased

TABLE I.

Qualitative Tests for Acetaldehyde Obtained at Different Stages in Fermentation.

Flask No.	Sodium sulfite.	On culture.				On distillate of the culture.
		3 days.	5 days.	7 days.	10 days.	10 to 12 days.
	per cent					
1	1	None.	Fair.	Good.	Strong.	Strong.
2	2	"	Slight.	"	"	"
3	3	"	"	Fair.	"	"

with the age of the culture until after 8 days a deep blue color extending to the bottom of the tube was obtained. In all the experiments that follow, the formation of aldehyde was traced by means of the Rimini test. The distillate obtained in the quantitative procedure was also tested for aldehyde by the Rimini test and in some cases with Schiff's reagent. A typical example of the results obtained from qualitative tests is given in Table I. Although these tests may be made on the cultures direct they are far better when used on the distillate. The amount of aldehyde formed was determined at the end of 7 days and again at the end of 12 days. On the second analysis a small increase was found in only one flask, No. 3. From this and other data (see Table II), it is apparent that aldehyde production is most active between the 2nd and 5th days, a period which

corresponds approximately to the time of greatest activity in the fermentation. The quantity of aldehyde fixed increases with the amount of sulfite present (see Table III); *e.g.*, with 1 per cent of sulfite the aldehyde fixed is 0.028 gm. per 100 cc. of culture and with 3 per cent of sulfite this has increased to 0.077 gm. At the end of the fermentation period, the control, Culture 1, to which no sulfite was added, and Cultures 3 and 4 were analyzed for alcohol, volatile acid, and non-volatile acid in addition to aldehyde. A high alcohol and low acid production is the normal type of fermentation with Culture 26. When sulfite is introduced, some of the intermediate compound acetaldehyde is fixed and the production

TABLE II.

Rate of Acetaldehyde Production by the Fermentation of Glucose. Calculated for 100 Cc. of Culture.

Age of culture.	Acetaldehyde.	Ethyl alcohol.	Volatile acid as acetic.
days	*gm.*	*gm.*	*gm.*
2	0.025		
4	0.046		
6	0.053		
11	0.065		
15	0.063		
20	0.068	0.075	0.232

Unfermented sugar 1.65 per cent.

of alcohol is decreased from 0.281 to 0.139 gm. per 100 cc. of culture. Coincident with the aldehyde and low alcohol production there is a rise in the volatile acid from 0.036 gm. in the control to 0.148 gm. in the flask containing 3 per cent of sodium sulfite. The volatile acid is evidently an oxidation product with which should be paired some reduction product, unless the hydrogen which is one of the gases formed under these conditions can account in some type of reaction for the oxygen used in the oxidation. Insufficient data exist to determine whether there is any relation between the hydrogen and the volatile acid. The complete data are given in Table III.

In order to have sufficient aldehyde to form the hydrazone with *p*-nitrophenylhydrazine a 2,000 cc. culture was prepared

The culture contained 3 per cent of glucose and 4 per cent of sodium sulfite. Aliquots were withdrawn at different intervals during the fermentation and the aldehyde was determined, as shown from the figures of Table II. The aldehyde content increases steadily up to the final analysis but most of it is formed by the end of 6 days. The low alcohol and high volatile acid production noted in Table III were repeated in this experiment.

The unfermented sugar was determined by the Ost method (25), since the Ost copper solution is not reduced to any extent by acetaldehyde as is the case with Fehling's solution. Glucose may, therefore, be determined in the presence of acetaldehyde by

TABLE III.

Acetaldehyde Production from Glucose.

Calculated for 100 Cc. of Culture.

Flask No.	Sodium sulfite in culture.	Age of culture.	Acet-aldehyde.	Ethyl alcohol.	Volatile acid as acetic.	Non-volatile acid as lactic.
	per cent	*days*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.0	12	0.006	0.281	0.036	0.000
2	1.0	7	0.028			
3	2.0	7	0.061			
3	2.0	12	0.045		0.112	0.010
4	3.0	7	0.061			
4	3.0	12	0.077	0.139	0.148	0.078

this method. The unfermented glucose amounted to 1.65 per cent which indicates that about one-half the glucose had been fermented. The data are given in Table II.

The remainder of the culture was treated with the required amount of barium chloride and the precipitated barium sulfite removed by filtration. To prevent loss of aldehyde during the filtration, this operation was carried on in a large refrigerator and the receiving flask was surrounded by a mixture of ice and salt. The clear solution thus obtained could be distilled without the foaming that occurs when the barium sulfite is not removed. About 1,500 cc. of filtrate were distilled with steam until the last portions of the distillate gave no test for aldehyde. The distillate was redistilled and collected in a 3 per cent solution of *p*-nitrophenylhydrazine dissolved in 50 per cent acetic acid.

About one and one-half times the theoretical quantity of hydrazine required to combine with the aldehyde was used. When the first few drops of distillate reached the receiving flask, a curdy yellow precipitate of acetaldehydehydrazone was formed. The distillation was continued until about 300 cc. of distillate had been collected. The vapors passing over at this time gave a negative Rimini test. After standing over night in the ice box, the hydrazone was filtered off with suction, washed with a little cold water, and dried over sulfuric acid. The melting point of the crystals was found to be 125°C. They were dissolved in 95 per cent alcohol and recrystallized. The melting point of the recrystallized hydrazone was found to be 128°C. A hydrazone prepared from acetaldehyde gave the same melting point. The melting point of acetaldehyde-*p*-nitrophenylhydrazine is given as 128.5°C.

TABLE IV.

Determination of Nitrogen in Acetaldehyde-p-Nitrophenylhydrazone.

Method.	No. of determinations.	Nitrogen.
		per cent
Kjeldahl, modified to include nitrates	5	10.6 to 21.2
Milbauer	1	3.8
" modified	3	23.15 to 23.37
Dumas combustion	1	23.8
Theory for $C_8H_9N_3O_2$	1	23.46

In order to check the evidence obtained from the melting point, the nitrogen content of the hydrazone was determined by the Dumas combustion and a modified Kjeldahl procedure. At first much difficulty was experienced in determining the percentage of nitrogen in the hydrazone by the Kjeldahl method. By increasing the amount of potassium persulfate, the time of digestion, and by adding potassium permanganate, Milbauer's (26) modified Kjeldahl method gave results that checked with the Dumas combustion method and with theory. To indicate how the figures varied, a summary of the results obtained are given in Table IV. The melting point and nitrogen content of the hydrazone and the qualitative color tests establish, without doubt, the fact that acetaldehyde is formed.

Acetaldehyde from Xylose.

Since Culture 26 is a rapid pentose fermenter, it seemed probable that acetaldehyde would be formed in the fermentation of xylose by this organism. Experiments were made with test-tube cultures to which were added varying amounts of sodium sulfite. In nearly every case, a strong test for aldehyde was noted. A flask containing 300 cc. of 2 per cent xylose-yeast water medium was inoculated and 15 cc. of a 20 per cent sodium sulfite solution were added at the time of inoculation. At the end of 1, 2, and 6 days, additional portions of sulfite were added until the final concentration of sulfite reached 3.5 per cent. At the end of 5 days, a 50 cc. aliquot was taken and the acetaldehyde determined. Successive determinations were made at the end of 7, 11, and 15 days. Alcohol, volatile acid, and non-volatile acid were also determined at the time of the last analysis. As was found in the fermentation of glucose, the aldehyde continues to increase to the end of the fermentation. The quantity of aldehyde is more than double that formed from glucose, and is the maximum amount obtained in any of the experiments. As already noted in the case of glucose, an unusually high production of acetic acid was found. The data are given in Table V.

TABLE V.

Production of Acetaldehyde by the Fermentation of Xylose. Calculated for 100 Cc. of Culture.

Age of culture.	Acetaldehyde.	Ethyl alcohol.	Volatile acid as acetic.	Non-volatile acid as lactic.
days	*gm.*	*gm.*	*gm.*	*gm.*
5	0.088			
7	0.102			
11	0.147			
15	0.157	0.074	0.486	0.150

Unfermented sugar 0.938 gm.

Acetaldehyde Production by Bacillus acetoethylicum.

This organism possesses the power of fermenting a wide range of carbohydrates among which is xylose. One of the conspicuous products of its activity is acetone. Under suitable conditions

this represents about 8 to 10 per cent of the carbohydrate in the culture (21). Besides acetone, formic and acetic acids, ethyl alcohol, lactic acid, and carbon dioxide are produced (27). The formation of acetaldehyde is of special interest in relation to the production of acetone. The reactions whereby the acetone is formed are not clearly understood but in all probability its production is associated with the condensation of the acetaldehyde

TABLE VI.

Production of Acetaldehyde in the Fermentation of Starch by Bacillus acetoethylicum in the Presence of Sulfites.

Calculated for 100 Cc. of Culture.

Experiment.	Flask No.	Sulfite.	Amount of sulfite.	Age of culture.	Acetaldehyde.
			per cent	*days*	*gm.*
	1		1.0*	14	0.018
A	2	Na_2SO_3	2.0*	14	0.013
	3		3.0*	15	0.020
	1		1.0*	12	0.010
B	2	Na_2SO_3	2.0*	12	0.027
	3		3.0*		—†
	1		1.0‡	7	0.012
				10	0.028
C	2	$CaSO_3.2H_2O$	2.0‡	9	0.023
				13	0.042
	3		3.0‡	13	0.000

* Anhydrous Na_2SO_3.

† Negative qualitative test, not analyzed.

‡ Calculated as $CaSO_3$.

and subsequent processes of oxidation and decarboxylation. As a tentative suggestion these changes may be represented as follows:

$$2CH_3.CHO = CH_3CHOH.CH_2CHO$$
$$CH_3CHOH.CH_2CHO + O_2 = CH_3-CO.CH_2COOH + H_2O$$
$$CH_3CO.CH_2COOH = CH_3.CO.CH_3 + CO_2$$

Reactions similar to the above are known to take place in fermentations and have been proposed by Neuberg and others (28) to explain the formation of different products. Reilly and his associates (29) have proposed somewhat similar reactions for the

production of acetone by *Bacillus granulobacter pectinovorum*. The production of 2.3–butylene glycol and acetylmethylcarbinol was explained by Harden and Norris (30) on a similar basis. In their work on the production of butyric acid by *Bacillus butylicus* from glucose and glycerol, Buchner and Meisenheimer (19) suggested that acetaldehyde was first formed from the glycerol and was then condensed to aldol and subsequently reduced or oxidized to form butyl alcohol and butyric acid, respectively. The importance of these balanced processes of reduction and oxidation in the organism has been emphasized by Palladin in a recent publication. Neuberg's work on the decarboxylation of pyruvic acid is so well known as scarcely to need mention.

In the experiments with *Bacillus acetoethylicum*, three series of flasks were prepared at different times with varying amounts of sodium or calcium sulfite as the fixative. The results obtained are given in Table VI, and are of the same general character as those obtained with the previous organism although only about one-half as much aldehyde was fixed. An increase of aldehyde results from increasing the fixative up to the limit of tolerance for sulfite by the bacteria. Calcium sulfite was rather more effective than sodium sulfite, as about one-half more aldehyde was obtained from the cultures where calcium sulfite was used.

Acetaldehyde from Xylose by Bacillus acetoethylicum.

In this experiment a peptone-phosphate medium containing 2.0 per cent of xylose was used. In one culture, sodium sulfite was used as the fixative agent and in another, calcium sulfite.

TABLE VII.

Production of Acetaldehyde in the Fermentation of Xylose by Bacillus acetoethylicum.

Calculated for 100 Cc. of Culture.

Sulfite.	Amount of sulfite.	Age of culture.	Acetaldehyde.
	per cent	*days*	*gm.*
Na_2SO_3	2.0	8	0.030
Na_2SO_3	2.0	12	0.079
$CaSO_3.2H_2O$	1.5*	12	0.011

* Calculated as $CaSO_3$.

As the sodium sulfite contained considerably less sodium sulfate than that used in the other experiments, the amount added was correspondingly less. The cultures were analyzed at varying intervals during the fermentation and were found to contain a much larger amount of aldehyde than was obtained from the glucose cultures. Similar high yields of acetaldehyde from xylose have already been noted in the fermentation of xylose by Culture 26. Sodium sulfite proved far more efficient as a fixative of aldehyde than calcium sulfite. The data are given in Table VII.

Acetaldehyde Production by Lactobacillus pentoaceticus.

This group of bacteria is rather sensitive to alkali, as was discovered from the results of neutralizing actively fermenting cultures. If, inadvertently, a slight excess of alkali was added to

TABLE VIII.

Production of Acetaldehyde by Lactobacillus pentoaceticus. Calculated for 100 Cc. of Culture.

Flask No.	Sulfite in culture.	Age of culture.	Acetaldehyde.
	gm.	*days*	*gm.*
2	1.0*	14	0.029
3	2.0*	13	0.017
4	0.5*	15	0.000
5†	1.0*	14	0.063
6	1.0‡	12	0.008
7	2.0‡	10	0.016

* Sodium sulfite.

† Carbon dioxide produced was 0.025 gm.

‡ Calcium sulfite.

the culture, growth ceased and was not resumed until a small quantity of acid was added. Therefore they do not lend themselves readily to experimentation in a sulfite medium. In the first series, a medium containing 2 per cent of glucose in yeast water was inoculated with the pentose fermenter No. 41–11 and, after 24 hours, enough sterilized sodium sulfite was added to give a concentraton of 1 and 2 per cent respectively in the two flasks.

In a second series, the same percentage of calcium sulfite was added as a fixative. Direct qualitative tests on the culture

showed only traces of aldehyde present. The strongest tests were obtained from the calcium sulfite series but the tests were only fair. After from 10 to 14 days, the cultures were analyzed for aldehyde, alcohol, volatile acid, and in a few cases for non-volatile acid. Only small quantities of these products were obtained. The carbon dioxide evolved from one of the flasks, No. 5, was determined and only 0.025 gm. per 100 cc. of culture was found. These results, coupled with strong reduction tests for glucose, showed that but little fermentation had taken place. The cause for this is probably due to the alkalinity of the sulfite medium or the toxic action of the sulfite on the bacteria. The data obtained for aldehyde are given in Table VIII.

The Fermentation of Pyruvic Acid.

According to Neuberg's theory, pyruvic acid is one of the intermediate products in alcoholic fermentation. The pyruvic acid then undergoes decarboxylation and acetaldehyde results. In the presence of sodium sulfite, Neuberg and Reinfurth (1) obtained 14 per cent of the theoretical yield of acetaldehyde, which would result if all the pyruvic acid had been completely fermented. The pentose-fermenting bacteria, employed in this study, ferment pyruvic acid to a greater or less extent. This has been shown in several ways: first, by the fermentation of the sodium salt of the acid and titration of the sodium bicarbonate formed; second, by the addition of dilute solutions of pyruvic acid to the fermenting culture and determination of the destruction of the free acid.

In the first experiment a 2 per cent solution of pyruvic acid was prepared, neutralized with solid sodium hydroxide, and then diluted to 1 per cent by the addition of double strength yeast water. This medium was then sterilized by passing it through a Pasteur-Chamberland filter, since a destruction of the pyruvate results if the solution is sterilized by heat. The solution was pipetted into large test-tubes and incubated for 5 to 6 days to determine its sterility. If no growth took place, the tubes were then inoculated, and sterilized brom-cresol purple was added. After a few days, a film of bacteria appeared on the surface, gas was evolved, and the solution became strongly

alkaline. The above evidences of growth were very marked in the case of Culture 26 and *Bacillus acetoethylicum*, but were slight in the tubes inoculated with *Lactobacillus pentoaceticus*. The slight fermentation with the last named organism is probably

TABLE IX.

Fermentation of Sodium Pyruvate by Pentose-Fermenting Bacteria.

Organism.	Tube No.	0.1 N sodium pyruvate present.	0.1 N alkali formed.
		cc.	cc.
Control	1	11.0	0.25
	2	11.0	0.60
No. 26	1	11.0	5.35
	2	11.0	6.60
B. acetoethylicum	1	11.0	1.50
	2	11.0	2.20
Lactobacillus pentoaceticus, No. 41–11	1	11.0	0.65
	2	11.0	1.22

In Tubes 1, pyruvic acid prepared in the laboratory was used.
In Tubes 2, pyruvic acid prepared by Eastman Company was used.

TABLE X.

Fermentation of Pyruvic Acid.

Tube No.	0.1 N acid.		
	Control.	Culture 26.	Culture 41-11.
	cc.	cc.	cc.
1	3.50	0.15	2.15
2	3.90		3.10
3	4.30	0.20	3.30
4	4.70	0.20	3.70
5	5.10	1.60	4.00

due to its sensitiveness toward alkali. It is essentially an acid producer and does not withstand much alkali. The conclusions drawn from the appearance of the tubes during the incubation are borne out by the titrations at the end of the fermentation. If we judge from the alkali developed, about one-half the pyru-

vate had been fermented by No. 26, about one-seventh by *Bacillus acetoethylicum*, and only a slight amount in the case of *Lactobacillus pentoaceticus*. The titration data are given in Table IX.

In a second experiment two series of large test-tubes containing varying amounts of yeast water were used. Sterilized pyruvic acid was added to these tubes from time to time and at the end of the incubation the unfermented acid was determined by titration. One series was left uninoculated and served as controls, a second series was inoculated with Culture 26, and a third with the pentose fermenter No. 41-11. Almost complete destruction of pyruvic acid resulted in the tubes inoculated with Culture 26 while only a small decrease of acid occurred in the tubes inoculated with Culture 41-11. The data obtained from the titrations are given in Table X. These two experiments showed that pyruvic acid and its sodium salt could be fermented.

An attempt was therefore made to ferment sodium pyruvate in the presence of sodium sulfite. To 100 cc. of 1 N pyruvic acid were added 80 cc. of 1 N sodium carbonate and 20 cc. of 1 N sodium sulfite, and this solution was made up to 1,000 cc. with yeast water. This medium is then equivalent to a 0.1 N solution of pyruvic acid and contains the sulfite in the form of the acid sulfite. It was sterilized by passing through a Chamberland filter and 18 cc. portions were pipetted into sterile fermentation tubes. After the tubes were incubated for a few days and no growth developed, they were inoculated in duplicate with Cultures 26, 41-11, and 118-8. One tube was left uninoculated as a control. A second series was set up where twice as much sulfite was added to the medium. The day after inoculation about 0.5 cc. of gas had accumulated in the tubes inoculated with Culture 26, and after 4 days this amounted to about 4 cc. The amount of gas collected in the tubes inoculated with Cultures 41-11 and 118-8 was never more than 1 cc. The small amount of gas accumulating is what would be expected since sodium bicarbonate is formed by the fermentation of sodium pyruvate. At the end of the fermentation period the carbon dioxide in the tubes was absorbed with sodium hydroxide. In the tubes inoculated with Culture 26 about one-half the gas remained unabsorbed and gave a test for hydrogen with a lighted splinter. The cultures were analyzed for their content of carbon dioxide by

means of the Van Slyke apparatus and the data thus obtained are given in Table XI. In all the cultures the production of carbon dioxide is clearly indicated; the maximum was noted in the case of Culture 26. If the carbon dioxide found originated entirely by the decarboxylation of the pyruvate, the possible maximum would be 0.080 gm. In Tube 2 of Series 1, 0.0604 gm. of CO_2 was found.

TABLE XI.

Fermentation of Sodium Pyruvate in the Presence of Sodium Sulfite.

Tube No.	Carbon dioxide produced by		
	Culture 26.	Culture 41-11.	Culture 118-8.
	gm.	*gm.*	*gm.*
Series 1.			
Control.	0.0016	0.0016	0.0016
1	0.0385	0.0172	0.0227
2	0.0604*	0.0174	0.0130
Series 2.			
Control.	0.0017	0.0017	
1	0.0457	0.0167	
2	0.0441		

* Mercury added to retain gas.

SUMMARY.

In the presence of a fixative, acetaldehyde is formed in the fermentation of carbohydrates by three types of pentose-fermenting bacteria: an organism related to the colon-aerogenes group, Culture 26; *Bacillus acetoethylicum;* and *Lactobacillus pentoaceticus.* Aldehyde is produced from glucose and starch but the maximum yield is obtained from xylose. The amount formed ranges from 0.020 to 0.157 gm. per 100 cc. of culture containing 2 per cent of the carbohydrate. The acetaldehyde is fixed in the process of fermentation by means of sodium sulfite or calcium sulfite. The quantity of aldehyde found is proportional to the amount of fixative used. Because of the alkalinity of sodium sulfite, the amount that can be added is limited. Sodium sulfite was more effective than calcium sulfite as a fixative for the aldehyde.

The production of acetaldehyde is correlated with the production of alcohol. An increase in the former results in a decrease in the latter. A high production of volatile acid is associated with the formation of acetaldehyde. These results emphasize the intimate relations between the balanced processes of reduction and oxidation that exist in a fermenting solution.

The production of acetaldehyde by the acetone-forming organism, *Bacillus acetoethylicum*, is of particular interest since it is possible that through the condensation of the aldehyde and subsequent processes of oxidation and decarboxylation the acetone is formed.

An attempt to correlate the production of acetaldehyde with Neuberg's theory, that pyruvic acid is an intermediate product in sugar fermentation, did not give conclusive results. Pyruvic acid and its sodium salt are fermented to a greater or less extent by all three types of bacteria. The aerogenes-like bacteria fermented it readily, whereas *Lactobacillus pentoaceticus* gave but a slight fermentation. Among the products formed are carbon dioxide, hydrogen, and volatile acid. These products were also formed when the pyruvic acid was fermented in the presence of sodium sulfite, but neither qualitative nor quantitative methods showed the presence of acetaldehyde.

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THE ESTIMATION OF CHLORIDES IN BLOOD.

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Renewed interest in blood chlorides has been aroused by Allen,[1] who has endeavored to show that salt retention is the cause of pure hypertension, and also of the hypertension in many cases of kidney disease. The estimation of this constituent has been most often conducted on the plasma, rather than on whole blood, but there would now appear to be some question as to the reliability of plasma analysis in this connection unless the plasma is immediately separated from the corpuscles. In any case a significant change in the blood chlorides should definitely affect the chloride content of whole blood as well as that of the blood plasma. The practical use of various laboratory tests of diagnostic import has ordinarily kept pace with their reliability and simplicity. That the estimation of the blood chlorides has not received more clinical consideration must be ascribed either to lack of appreciation of the possible value of the test, or to the difficulties in technique. A method of chloride estimation is described below which we believe contributes to the simplicity and rapidity of the test, without impairing its accuracy.

Most American workers who have studied this question have employed the iodimetric method of McLean and Van Slyke,[2] or some modification of it. With their original method the blood proteins were removed with the aid of heat, acetic acid, magnesium sulfate, and blood-charcoal. The chlorides were then precipitated in acid solution with silver nitrate and the excess was titrated with iodine, using starch as an indicator. Owing to

[1] Allen, F. M., *J. Am. Med. Assn.*, 1920, lxxiv, 652.

[2] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

the difficulty in securing suitable blood-charcoal, Harding and Mason[3] modified the method by employing copper sulfate and alkali for the removal of the protein. Later in the same year Foster[4] utilized *m*-phosphoric acid to remedy the same difficulty. Almost simultaneously with the publication of the McLean and Van Slyke method, Myers and Fine[5] described a technique of chloride estimation and later reported a few observations on the blood chlorides,[6] although these were not then discussed. With this method the proteins were coagulated in the presence of five volumes of 0.01 N acetic acid with a total dilution of 1 to 10, the last trace of protein being removed with a few drops of colloidal iron. The chlorides were then titrated either according to the Volhard-Arnold method, using solutions one-tenth the strength of those employed for urine, or directly with the Mohr method in this practically neutral solution. The disadvantage of this method was in the difficulty of obtaining chloride-free colloidal iron. 3 years later Rappleye[7] likewise made use of the Volhard titration for the estimation of chlorides in blood plasma. Still later Van Slyke and Donleavy[8] greatly simplified the McLean-Van Slyke technique by introducing sufficient picric acid into the silver nitrate-nitric acid reagent to precipitate completely the proteins and chlorides in one operation. Although this method proved perfectly satisfactory for plasma, Austin and Van Slyke[9] later noted that it gave too high results with whole blood, apparently as the result of the silver being bound by some constituent in the corpuscles. To overcome this difficulty for whole blood they now first precipitate the proteins with saturated picric acid solution. After filtration the chlorides are then estimated essentially as in the McLean-Van Slyke method.

[3] Harding, V. J., and Mason, E. H., *J. Biol. Chem.*, 1917, xxxi, 55.

[4] Foster, G. L., *J. Biol. Chem.*, 1917, xxxi, 483.

[5] Myers, V. C., and Fine, M. S., Chemical composition of the blood in health and disease, New York, 1915, 34. also Myers, V. C., *Post-Graduate*, 1915, xxx, 38.

[6] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xx, 391.

[7] Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.

[8] Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

[9] Austin, J. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 345.

In connection with the present study we have tried a number of methods of protein precipitation, including the tungstic acid method of Folin and Wu.[10] None of these has proved so satisfactory in our hands as the use of picric acid recommended by Van Slyke and Donleavy. Several years ago an attempt was made to utilize the picric acid filtrate employed in the estimation of sugar and creatinine for the chlorides, but at that time this method was not believed to be so satisfactory as the heat and acetic acid procedure. Our present observations, however, confirm the findings of Austin and Van Slyke as to the reliability of picric acid as a blood protein precipitant in the estimation of chlorides. Furthermore, they indicate that it makes little difference in the results obtained whether the blood is diluted 1 to 20 as in their technique, or 1 to 10 or 5. Since the 1 to 5 dilution is employed in the estimation of sugar[11] and creatinine,[12] the chloride estimation may be made on the same filtrate. We have preferred to adhere to the Volhard titration partly because this allows the use of the same solutions (diluted) employed in the Volhard-Harvey[13] method for urine, and partly because of the rather rapid deterioration of the starch indicator used in the iodimetric method. When the starch solution is fresh the endpoint is very sharp, sharper than in the thiocyanate titration, although with a little experience the latter is readily recognized. Many workers have complained of difficulties with the silver chloride filtration. We believe that the quickest and most satisfactory method of removing this rather troublesome precipitate is with the aid of the centrifuge as originally suggested.[5]

To summarize the advantages of the technique described for blood chlorides, the estimation may be carried out: (1) on the same filtrate as used in the estimation of creatinine and sugar; (2) with the same reagents (diluted) as commonly employed for chloride estimation in urine (Volhard-Harvey method); and (3) considerable time may be saved by removing the troublesome silver chloride precipitate by centrifuging rather than by filtration.

[10] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
[11] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.
[12] Myers, V. C., *J. Lab. and Clin. Med.*, 1920, v, 566.
[13] Harvey, S. C., *Arch. Int. Med.*, 1910, vi, 12.

Method for Chloride Estimation in Blood.

When blood chlorides only are to be estimated there are some advantages in employing the 1 to 10 dilution and this technique will be described first.

Solutions Required.

1. Silver nitrate of such strength that 1 cc. is equivalent to 1 mg. of sodium chloride, 2.904 gm. to 1,000 cc. It may be prepared by a 1 to 10 dilution of the silver nitrate employed in the Volhard-Harvey method[13] for urine.

2. Acid ferric alum indicator prepared by dissolving 100 gm. of crystalline ferric ammonium sulfate in 100 cc. of 25 per cent nitric acid and adding four parts of distilled water. This is one-fifth the strength employed for urine.

3. Ammonium thiocyanate of such strength that 2 cc. are the equivalent of 1 cc. of the above silver solution, or one-twentieth of the strength employed for urine. It contains approximately 0.65 gm. of the thiocyanate to 1,000 cc.

To simplify the test further it is possible to combine Solutions 1 and 2, two parts of No. 1 and one part of No. 2 being used with the 1 to 10 dilution of the blood, this combined solution being further diluted with one part of distilled water when the 1 to 5 blood dilution is employed.

3 cc. of whole blood (or plasma) are added to 27 cc. of distilled water in a 50 cc. centrifuge tube. About 0.5 gm. of dry picric acid is then added and the mixture stirred until protein precipitation is complete and the whole mixture turns a bright yellow color. The precipitate is now thrown down by centrifuging for a few minutes at moderate speed and the clear supernatant fluid decanted into a clean dry beaker. (If any particles remain suspended the fluid should be filtered.)

20 cc. are then pipetted into a clean dry 50 cc. centrifuge tube and 20 cc. of standard $AgNO_3$ solution, of such strength that 1 cc. is equivalent to 1 mg. of NaCl (Solution 1), and 10 cc. of the dilute acidified ferric alum indicator added (Solution 2). The contents are stirred to insure thorough mixing and the AgCl preprecipitate is thrown down in the centrifuge. The clear supernatant fluid is decanted into a clean, dry beaker and 20 cc. portions are pipetted into each of two small porcelain evaporating dishes for duplicate titrations.

The titration is made with ammonium thiocyanate solution of such strength that 2 cc. are equivalent to 1 cc. of the $AgNO_3$ solution (Solution 3). The end-point is definite and consists of the first permanent tinge of reddish brown which extends throughout the mixture. Some experience may be necessary before the end-point is always recognized, but thereafter there need be no difficulty in obtaining exact duplicate titrations. Passing the end-point by one drop will introduce an error ordinarily of about 0.5 per cent in estimating chlorides in 100 cc. of blood or plasma.

When it is desired to utilize the 1 to 5 picric acid filtrate employed for sugar and creatinine, 5 cc. (equivalent to 1 cc. of blood) are employed, instead of 10 cc., for a single determination. If this method is to be followed regularly it is desirable to dilute the indicator solution further by one-half. In this case 5 cc. of the filtrate are pipetted into a 25 or 50 cc. centrifuge tube and 10 cc. of both the standard silver nitrate and ferric alum indicator added (or 20 cc. of the mixture referred to under "Solutions required"). After centrifuging titration is carried out on 20 cc. of the supernatant fluid as described above.

The calculation is $10 - \left(\frac{\text{Titer}}{2} \times \frac{5}{4}\right) \times 100 =$ mg. of NaCl in 100 cc. of whole blood or plasma.

With the above method it has been found possible to determine the sodium chloride content of pure solutions with an error of less than 1 per cent, and sodium chloride added to blood may be recovered with a similar degree of accuracy. A parallel series of results obtained with the Austin-Van Slyke method and the method described is given in Table I. As will be noted there is very close agreement between the two methods. In the case of the method described it apparently makes little difference whether a dilution of 1 to 5 or 1 to 10 is employed. All the observations reported in the table were obtained on whole blood.

In his first paper dealing with the chlorides of the blood plasma McLean[14] considered the influence of the plasma being allowed to stand in contact with the cells. He concluded that the change took place very slowly and that it was necessary only to centri-

[14] McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212.

fuge within 2 to 3 hours to avoid the danger. In their first paper on the plasma bicarbonate Van Slyke and Cullen[15] called attention to the effect which carbonic acid changes in whole blood might have on the chloride content of the plasma, a loss in bicarbonate resulting in an increase in the plasma chloride. This observation has recently been confirmed and extended by Fridericia,[16] who states that estimation of the chlorides in the

TABLE I.

Comparison of Results Obtained with Austin-Van Slyke Method and Method Described.

Specimen.	NaCl per 100 cc.			Remarks.
	Austin-Van Slyke method.	Method described.		
		1:10 dilution.	1:5 dilution.	
	mg.	*mg.*	*mg.*	
1. J. J.	456	456	462	Normal.
2. M. B.	487		487	"
3. S. D.	487	481	500	"
4. I. S.	475	462	481	"
5. W. S.	447	444	444	"
6. R. I.	462	462	467	"
7. D.	475		475	Essential hypertension.
8. R. K.	456	460	456	Slight nitrogen retention.
9. F. M.	587	584	584	Advanced nephritis.
10. J. C.	531	537	537	" "
11. M. D.	475		469	" "
Average.	485		487	

plasma or serum from blood which has been kept in open receivers must give too high results, because chlorides have passed into the plasma (or serum) on account of the decreasing CO_2 tension. McLean, Murray, and Henderson[17] have likewise considered this question in a preliminary way. Observations bearing on this question are given in Table II. In taking the control

[15] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.

[16] Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.

[17] McLean, F. C., Murray, H. A., Jr., and Henderson, L. J., *Proc. Soc. Exp. Biol. and Med.*, May 22, 1920.

specimens of blood under oil the technique of Van Slyke and Cullen[15] was followed. The subjects were normal, two being men and two women. In general it will be noted that standing increased the chloride content of the plasma. This being the case, results obtained on whole blood would appear to be more trustworthy than those obtained on plasma. Whole blood ordinarily contains about 100 mg. less NaCl per 100 cc. than plasma.

TABLE II.

Influence of Standing on Plasma Chloride.

Subject.	Chloride content of whole blood (NaCl per 100 cc.).	Chloride content of blood plasma (NaCl per 100 cc.).			
		Taken under oil and separated immediately.	Taken in open container and separated immediately.	Taken in open container and separated later.	
				Time.	NaCl.
	mg.	*mg.*	*mg.*	*hrs.*	*mg.*
1. J. S.	506	600	612	18	669
2. L. J.	475	587	644	2	612
3. H. C.	538	644	638	2	650
4. F. T.	538	612*		24	644

* After slow aeration for 5 min. the figure was 616 mg.

SUMMARY.

A method of chloride estimation in blood is described, in which the same picric acid filtrate employed for the estimation of sugar and creatinine, and the same solutions (diluted) ordinarily employed for the estimation of chlorides in urine are utilized.

RAPID COLORIMETRIC METHODS FOR THE DETERMINATION OF PHOSPHORUS IN URINE AND BLOOD.

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Several years ago, Taylor and Miller (1914) described a method for the determination of phosphates depending upon precipitation as ammonium phosphomolybdate and colorimetric estimation of the precipitated molybdenum by reduction with phenylhydrazine. Although this method has been quite extensively used, and other reducing agents have been proposed, the observation does not seem to have been made previously that certain reducing agents will reduce phosphomolybdic acid without affecting molybdic acid. By taking advantage of this selective reduction, the molybdenum present as phosphomolybdic acid can be colorimetrically determined in the presence of an excess of molybdic acid, avoiding the necessity of isolating the ammonium phosphomolybdate.[1]

We have found that hydroxylamine and many phenols have this selective reducing action in weakly acid solution at room temperature and we have selected hydroquinone as the most suitable reducing reagent. The color produced by the reduction

[1] After this paper was prepared for publication, we were shown the proof of a paper recently published by Dr. Wu in this *Journal*, in which he refers to this specific reduction and proposes a tentative method for the determination of phosphate. While we knew that Dr. Wu was investigating phosphotungstic and phosphomolybdic acids, we were unacquainted with the details of his work and have derived no information from it. We demonstrated the reduction of phosphomolybdic acid and the non-reduction of molybdic acid by hydroxylamine in the presence of Dr. Wu several months before his paper was sent in for publication and stated that we intended to develop methods for the determination of phosphate on this basis. We, therefore, do not feel bound by his implied reservation of this field.

of the phosphomolybdic acid by the hydroquinone is intensified by making the solution alkaline and a greenish shade due to the presence of quinone is removed by the addition of sulfite. The alkali (carbonate) and the sulfite are added in one solution.

If hydroquinone is added to a weakly acid solution of molybdic acid, no phosphate being present, the mixture will be perfectly colorless when treated with alkaline sulfite, but, if phosphate is present, phosphomolybdic acid is formed and reduced giving a blue color. As little as 0.005 mg. of phosphorus as phosphate gives a distinct blue color in a volume of 100 cc. The reaction is the same as in the phenol determination of Folin and Denis (1915), except that in our case there is an excess of phenol, and in theirs an excess of phosphomolybdic acid.

In the actual determination, the solution to be determined is placed in one volumetric flask and a standard solution of potassium phosphate in another. Distilled (phosphate-free) water is added to make both solutions to the same volume, and molybdic acid and hydroquinone solutions are added to both. After standing 5 minutes, the alkaline sulfite solution is added, the flasks are made up to volume, and the colors compared in the colorimeter in the usual manner after further standing of 5 to 10 minutes. It is possible that not all the phosphomolybdic acid is reduced by this process, but the color is strictly proportional to the amount of phosphate present, over a wide range of concentration. The unknown and the standard should have nearly the same acidity, volume, and temperature during the reduction with hydroquinone in acid solution, since variation in these factors has some effect on the final depth of color.

We have developed methods for the determination of inorganic, organic, and total phosphorus in urine and inorganic and total acid-soluble phosphorus in whole blood, plasma, and serum, using the solutions given below.

Molybdic Acid Solution.—50 gm. of pure ammonium molybdate are dissolved without heat in 1 liter of phosphate-free normal sulfuric acid. 5 cc. of this solution, treated with an equal amount of the hydroquinone solution and, after standing 5 minutes, with 25 cc. of the carbonate-sulfite solution, should give an absolutely colorless mixture. If the ammonium molybdate contains phosphate, it may be purified as follows: 150 gm. of

ammonium molybdate are dissolved in 1 liter of water and added to 375 cc. of concentrated nitric acid (specific gravity 1.42) which has been diluted to 1 liter. 200 gm. of ammonium nitrate are dissolved in this mixture and the whole is allowed to stand several days in a warm place. After the precipitated ammonium phosphomolybdate is filtered out, the filtrate is treated with two volumes of alcohol and then with ammonia until only faintly acid to litmus. The molybdate separates completely in a few minutes and is filtered out with suction, washed with 50 per cent alcohol, and dried.

This solution keeps indefinitely. The formation of a small amount of sediment does no harm; it may be removed by decantation.

Hydroquinone Solution.—20 gm. of pure hydroquinone are dissolved in 1 liter of phosphate-free water and 1 cc. of concentrated sulfuric acid is added. On standing, this solution becomes colored due to the formation of some quinone. A moderate amount of color does no harm as the quinone is reduced in the determination by the alkaline sulfite solution. The solution should be kept tightly stoppered to reduce the amount of oxidation by the air.

Carbonate-Sulfite Solution.—To 2,000 cc. of a 20 per cent solution of Na_2CO_3 are added 75 gm. of sodium sulfite dissolved in 500 cc. of water. The mixture is filtered. The presence of a moderate amount of phosphate in this solution does no harm since phosphomolybdates are not formed in alkaline solution.

Stock Solution of Monopotassium Phosphate.—Pure monopotassium phosphate is finely ground and exposed in a desiccator over sulfuric acid for several days. Of this dry preparation, 4.394 gm. are dissolved and made up to 1 liter with phosphate-free water in an accurate volumetric flask. 1 cc. of this solution contains 1 mg. of phosphorus. The solution should be preserved with chloroform and kept tightly stoppered.

Standard Phosphate Solution for Urine.—Of the above stock solution of phosphate, 50 cc. are accurately measured into a 500 cc. volumetric flask and made up to volume with phosphate-free water. This solution should also be preserved with chloroform. If the solution becomes turbid or if molds appear, a new solution should be made up. 5 cc. of this solution contain 0.5 mg. of phosphorus.

Dilute Standard Phosphate Solution for Blood.—5 cc. of the stock phosphate solution are made up to 1,000 cc. and preserved with chloroform in the same manner as the standard solution for urine. 5 cc. of this solution contain 0.025 mg. of phosphorus.

Urine.

Inorganic Phosphate.—1 to 5 cc. of urine, according to concentration, are accurately measured into a 100 cc. volumetric flask and 25 cc. of phosphate-free water added. In a similar flask are placed 5 cc. of the standard phosphate solution for urine, also with 25 cc. of water. To both flasks are added 5 cc. of the molybdic acid solution and 5 cc. of the hydroquinone solution. After standing 5 minutes, 25 cc. of the carbonate-sulfite solution are added and the flasks made up to volume and mixed. The solutions are read after 5 to 10 minutes. While the color is not absolutely permanent, we have found that the standard and the unknown fade at almost exactly the same rate, and that readings made at the end of an hour give the same result as those made at the end of 5 minutes. However, the solution in the cup of the colorimeter does not fade at the same rate as that in the flask, and both cups of the colorimeter should be filled at the same time whenever a comparison is to be made.

If the urine is read against the standard set at 20 mm. the calculation is as follows:

$$\frac{10}{\text{Reading} \times \text{cc. urine used}} = \text{Gm. inorganic P per liter}$$

If an insufficient amount of water is added to the urine before adding the molybdic acid, certain urinary constituents are precipitated by the phosphomolybdic acid leading to slightly high results.

Since 1 to 4 cc. of urine are diluted to 100 cc. during the determination, the color of the urinary pigments seldom interferes with the determination. We have even determined urines containing bile by the above process. If the color of the urine is troublesome, it may be removed by adsorption. For this purpose we have found certain samples of "activated carbon," prepared by the National Carbon Company of Cleveland, to be satisfactory.

A carbon to be used for this purpose should be carefully tested by comparing determinations on the standard solution with and without treatment with carbon, to insure that phosphate is neither added nor removed.

Total Phosphorus.—1 cc. of urine is measured with an Ostwald pipette into a hard glass test-tube and 6 to 8 drops of concentrated sulfuric acid, 1 cc. of concentrated nitric acid, and a piece of quartz are added. The tube is cautiously heated until nitrous fumes no longer come off and the remaining drops of sulfuric acid are colorless. Care should be taken to avoid evaporating to dryness or pronounced overheating which may cause loss of phosphoric acid.[2] The remaining few drops of sulfuric acid are transferred to a 100 cc. volumetric flask with about 25 cc. of phosphate-free water. Into a similar volumetric flask, containing 4 to 6 drops of concentrated sulfuric acid, are measured 2, 3, or 5 cc. of the standard phosphate solution for urine, the amount being determined by the result found for inorganic phosphate. Having been brought to the same volume with phosphate-free water, the standard solution and urine are treated and compared as in the determination of inorganic phosphate. If the urine is compared with the standard set at 20 mm., the calculation is as follows:

$$\frac{2 \times \text{cc. standard used}}{\text{Reading}} = \text{Gm. total P per liter}$$

Organic Phosphorus.—As pointed out by Taylor and Miller (1914), it is not permissible to estimate organic phosphorus by subtracting inorganic from total phosphorus since the amount of organic phosphorus is so small. They recommend precipitation of the inorganic phosphate with barium and determination of the organic phosphorus in the filtrate. The technique is as follows: 20 cc. of the urine are measured into a 25 cc. volumetric flask, made just alkaline with powdered barium hydroxide, filled to the

[2] We have not found that the addition of cane sugar as recommended by Bloor has any influence on the amount of phosphorus obtained by our method. It is possible that phosphoric acid compounds of the type mentioned by Bloor might give the full amount of color by our method and yet fail to be precipitated by his strychnine molybdate reagent. On the other hand, pyro- and *m*-phosphoric acids do not form phosphomolybdic acid and so escape determination by our method.

mark with water, and filtered. The precipitate contains barium sulfate and phosphate. To remove the excess of barium, 20 cc. of this filtrate are measured into another 25 cc. flask, made just acid with dilute sulfuric acid, filled to volume, and filtered. An excess of sulfuric acid is to be avoided since it affects the final acidity of the determination. 1 cc. of this second filtrate is equivalent to 0.64 cc. of the original urine.

We have made a few determinations of organic phosphorus in the following manner: 10 cc. (equivalent to 6.4 cc. of urine) of filtrate prepared as above are evaporated with 8 to 10 drops of concentrated sulfuric acid to about 2 cc., and 2 cc. of concentrated nitric acid are added. The digestion is carried out as described under "Total phosphorus" and the residue transferred to a 25 cc. flask with 10 cc. of distilled water. In another similar flask are placed 5 cc. of the dilute standard solution for blood (equivalent to 0.025 mg. of P), 4 to 6 drops of concentrated sulfuric acid, and 5 cc. of water. To both flasks are added 1 cc. of the molybdic acid solution and 2 cc. of the hydroquinone. After 5 minutes, 10 cc. of the carbonate-sulfite solution are added, and the flasks made up to volume and read as usual, except that it is better to set the standard at 30 or 40 mm. instead of 20 mm. If the urine is read against the standard set at 40 mm., the calculation is as follows:

$$\frac{1{,}000}{\text{Reading} \times 6.4} = \text{Mg. organic P per liter}$$

Urines high in organic phosphorus may be read against a stronger standard. It is better, for most urines, to use the dilute standard described above, rather than to increase the amount of urine filtrate used, since the smaller the amount of urine, the easier is the digestion.

Determinations of organic phosphorus should be done on fresh urines, since, as pointed out by Mathison (1909), the values decrease on standing.

We have also determined the organic phosphorus in urine by precipitating the inorganic phosphate with magnesium citrate as described below, and estimating the phosphorus in the filtrate. The values thus obtained are slightly higher and probably more accurate than those obtained by the barium precipitation, but the

determination is not so convenient and requires at least 8 hours for complete precipitation.

Accuracy.—We attempted to check the results secured on urines by these methods against gravimetric determinations of inorganic phosphate. 50 cc. portions of urine were precipitated with magnesium citrate according to the technique of Mathison (1909) and filtered after standing over night. The paper and precipitate were dried over night at room temperature and weighed. The precipitate was then brushed off the paper and the paper reweighed as suggested by Jones (1916).[3]

Table I gives the values for inorganic phosphate obtained by this gravimetric process, by our colorimetric method, and by the uranium titration, using ferrocyanide as indicator. The results by the uranium titration are usually distinctly higher than by the other two methods. The colorimetric and gravimetric methods both give good duplicate determinations, but the colorimetric values are higher.

That the difference between the results by the gravimetric and colorimetric methods is due to too low results by the former, is indicated by the values in Table II. The determinations in this series were done just as in the first series except that the filter papers, after reweighing, were extracted with dilute sulfuric acid and the phosphate thus recovered was determined colorimetrically. The first column under gravimetric determination in Table II gives the result calculated from the weight of precipitate obtained, the second column gives the additional amount calculated from the precipitate adhering to the filter paper, and the third column gives the sum of these two. In this second series, the results by the colorimetric and the corrected gravimetric methods are in much better agreement than in the first series. The results by the uranium titration tend to be higher than those by the other methods, and this is especially true in the urines containing albumin and bile.

Although the filter paper (7 cm., Schleicher and Schull's No. 589, black ribbon) was as small as could be conveniently used, examination of the column of corrections shows that quite large

[3] This method of determination was suggested to us by Dr. C. H. Fiske of this department, to whom we are also indebted for other valuable suggestions.

TABLE I.

Inorganic Phosphate as P per Liter.

Urine.	Gravimetric.	Colorimetric.	Uranium.
	gm.	*gm.*	*gm.*
1. Normal mixed.		0.135	0.145
		0.135	
2. " "		0.119	0.127
		0.119	
3. " "	0.5568	0.555	0.562
	0.5564	0.553	
4. " "	0.2388	0.253	0.262
	0.2384	0.255	0.260
5. " "	0.1356	0.143	0.145
	0.1356	0.142	0.146
6. " "	0.2899	0.294	0.310
	0.2899	0.294	0.307
7. Sugar 1 per cent; $FeCl_3$+++.	0.3954	0.421	0.415
	0.3954	0.416	0.414
8. " 2 " " $FeCl_3$+++.	0.1911	0.216	0.213
	0.1915	0.218	0.210
9. No sugar; $FeCl_3$+++.	0.3706	0.406	0.392
	0.3713		0.389
10. Albumin +++.	0.4408	0.455	0.445
	0.4417		0.450
11. Normal mixed.	0.3000	0.308	0.313
	0.3012		
4a. "	0.2850	0.284	
8a. Sugar present.	0.1918	0.211	0.209

TABLE II.

Inorganic Phosphate as P per Liter.

Urine.	Gravimetric.			Colorimetric.	Uranium.
	Weighed.	Correction.	Corrected value.		
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
12. Normal mixed.	0.5739	0.0028	0.5767	0.584	0.564
	0.5693	0.0073	0.5766		0.570
	0.5703	0.0056	0.5759		
13. Sugar 2.3 per cent; $FeCl_3$++; albumin +.	0.2315	0.0090	0.2405	0.235*	0.263
					0.269
14. Sugar 0.1 per cent; $FeCl_3$−.	0.2169	0.0068	0.2237	0.227	0.225
	0.2134	0.0096	0.2230		0.224
14a. Same as No. 14, 2 days later.	0.2399	0.0084	0.2483	0.244	
15. Sugar 0.2 per cent; $FeCl_3$+++.	0.1244	0.0010	0.1254	0.124	0.127
	0.1254	0.0007	0.1261		
16. Sugar 1.5 per cent; $FeCl_3$++.	0.1840	0.0048	0.1888	0.188	0.193
	0.1858	0.0035	0.1893		0.195
17. Sugar 0.4 per cent; $FeCl_3$−; bile ++.	0.4649	0.0192†	0.4841	0.485	0.515
	0.4700	0.0167†	0.4867		
18. Normal mixed.	0.2599	0.0035	0.2634	0.258	0.269
	0.2599	0.0035	0.2634	0.260	0.271
19. " "	0.1524	0.0030	0.1554	0.157	0.162
	0.1532	0.0025	0.1557	0.158	0.162
20. " "	0.1636	0.0018	0.1654	0.166	0.169
	0.1641	0.0015	0.1656	0.167	0.170

* This value was obtained after clearing with carbon. The urine contained much mucus and was very turbid.

† The precipitate in this urine was extremely fine so that some of it passed through the filter. This was collected by centrifuging, and is included in the correction.

and variable amounts of precipitate adhere so firmly that they cannot be brushed loose. The amount of this adhering precipitate varies with different urines, though it is usually nearly the same in duplicate determinations, and it is perhaps dependent on variations in crystal size and shape in different urines. The urines having the largest corrections are those containing albumin and bile. When dealing with 50 cc. of urine, it is evident that satisfactory duplicate determinations by weighing magnesium ammonium phosphate are no guarantee of accuracy. However, the variations obtained with different urines and the low correction in certain urines (Nos. 15 and 20, Table II) suggest that with other material the adhering precipitate may be much less.

Blood.

For the determination of the inorganic and acid-soluble phosphorus in blood we have made use of trichloroacetic acid. The acid ammonium sulfate used by Bloor (1918) cannot be conveniently used with our method on account of the large amount of salts in the filtrate. The tungstic acid filtrates of Folin and Wu (1919) give consistently low results, probably due to the formation of phosphotungstic acid from the tungstic acid reagent and the inorganic phosphate of the blood and combination of this substance with the protein precipitate. The acetic-picric acid filtrates of Greenwald (1916) cannot be used for the determination of inorganic phosphate by our method on account of the interference of the color of the picric acid. Acetic-picric acid filtrates can be used for the determination of the total acid-soluble phosphorus if the picric acid is driven off as described by Greenwald and the residue digested with nitric and sulfuric acids. We have always found trichloroacetic acid to be free from phosphorus, but all samples should be tested.

We have found that a dilution of 1:5 is sufficient for normal plasma and gives the same result as a dilution of 1:10. If the plasma is suspected to be high, or if whole blood is used, we recommend a dilution of 1:10. 5 cc. of plasma are mixed with 15 cc. of distilled water in a 25 cc. volumetric flask and 5 cc. of 20 per cent trichloroacetic acid added with shaking. The flask is filled to the mark with water, mixed, and after 10 minutes is

filtered through a phosphate-free (acid-washed) filter. If whole blood is used, 5 cc. are laked by mixing with 35 to 40 cc. of distilled water in a 50 cc. volumetric flask and 5 cc. of 20 per cent trichloroacetic acid are added with shaking. The flask is made up to volume, and filtered after 10 minutes.

Inorganic Phosphate.—To 10 cc. of the trichloroacetic acid filtrate in a 25 cc. volumetric flask are added 1 cc. of the molybdic acid solution and 2 cc. of the hydroquinone solution. At the same time, molybdic acid and hydroquinone are added to a similar flask containing 10 cc. (equivalent to 0.05 mg. of P) of the dilute standard phosphate solution for blood, and either 1 or 2 cc. of 20 per cent trichloroacetic acid, according to whether a dilution of 1:10 or 1:5 has been used. After 5 minutes, 10 cc. of the carbonate-sulfite solution are added, and the flasks are made up to volume and mixed. The colors are compared after 5 to 10 minutes. If the unknown is read against the standard set at 20 mm., the calculation is

$$\frac{100}{\text{Reading}} = \text{Mg. P per 100 cc. (if 1:10 dilution was used)}$$

or

$$\frac{50}{\text{Reading}} = \text{Mg. P per 100 cc. (if 1:5 dilution was used)}$$

It may be necessary to use a stronger standard or less filtrate in bloods containing much phosphorus. The proper standard and amount of filtrate may be roughly estimated before the determination is made. A 5 cc. mark is made on two small similar test-tubes. In one of these tubes is placed 1 cc. of the dilute standard phosphate solution for blood and in the other, 1 cc. of filtrate. About 10 drops of molybdic acid and hydroquinone solutions are added to both tubes, and after a few minutes the tubes are filled to the mark with carbonate-sulfite solution. The amount of color may be roughly gauged and the strength of standard and amount of filtrate decided upon. Serum or plasma may be roughly estimated in the same manner before removing the proteins in order to judge the proper dilution to use.

The inorganic phosphate must be determined in the trichloroacetic acid filtrate as soon as possible after filtering since the acid hydrolyzes the organic phosphorus on standing, giving too high

results. After standing 24 hours, the values for inorganic phosphate and total acid-soluble phosphorus are nearly identical. One plasma, containing 9.6 mg. of acid-soluble phosphorus per 100 cc., gave the following values for inorganic phosphate: 30 minutes after filtering, 8.8 mg.; 18 hours after filtering, 9.4 mg. Another plasma, containing 3.5 mg. of acid-soluble phosphorus,

TABLE III.

Inorganic and Total Acid-Soluble Phosphorus as P per 100 Cc.

Specimen.	Inorganic phosphate.	Acid-soluble phosphorus.	
		Trichloro-acetic acid.	Acetic-picric acid.
	mg.	*mg.*	*mg.*
1. Plasma, human	3.5	5.0	
2. " "	3.9*	4.0	
3. Whole blood, beef	4.9	8.3	
	5.1	8.6	
4. " " "	5.9	11.6	
5. Plasma, human (hemolysis)	9.0	10.5	
6. " " (diabetic)	4.4	5.1	
7. " " "	4.4*	4.4	
8. " " "	4.5	5.0	
9. " " "	4.8†	4.7	4.8
10. " " (hemolysis)	8.8	9.8	9.6
11. Whole blood, beef	5.1	10.3	10.3
12. Plasma, human (diabetic)	3.2	3.6	3.5
13. " beef	8.0*	8.1	8.0
14. Same as No. 13 with added phosphate (40 mg. per 100 cc.)	47.7		

* These specimens were several days old when analyzed.

† The filtrate from this specimen stood 24 hours before the determination was carried out.

gave the following values for inorganic phosphate: 30 minutes after filtering, 3.2 mg.; 90 minutes after filtering, 3.4 mg.; 5 hours after filtering, 3.4 mg.

Total Acid-Soluble Phosphorus.—The total acid-soluble phosphorus is determined in a manner similar to the total phosphorus in urine. 10 cc. of the trichloroacetic acid filtrate, in a hard glass test-tube, are evaporated down to about 2 cc. with 6 to 8 drops of concentrated sulfuric acid and a piece of quartz. 1 cc.

of concentrated nitric acid is added and the digestion continued until all the nitric acid has been driven off and the remaining drops of sulfuric acid are clear. The residue is transferred to a 25 cc. volumetric flask with about 10 cc. of distilled water and treated as under inorganic phosphate. The standard to be used is determined by the value previously found for inorganic phosphate. 4 to 6 drops of concentrated sulfuric acid are added to the standard to balance that used in the digestion but no trichloroacetic acid is added, since all of this substance is volatilized during the digestion.

Results.—Table III gives some values obtained by these methods. The agreement between the trichloroacetic acid and picric acid filtrates is satisfactory. The ratio of inorganic to total acid-soluble phosphorus is about that usually found by other methods.

SUMMARY.

Rapid colorimetric methods are described for the determination of inorganic, organic, and total phosphorus in urine. While not so accurate as suitable gravimetric determinations, these methods are much more convenient and are sufficiently accurate for many purposes. The method for inorganic phosphate appears to be more accurate and rapid than the usual uranium titration.

Methods are also described for the determination of inorganic and acid-soluble phosphorus in blood.

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THE EFFECT OF DIET ON THE EXCRETION OF INDICAN AND THE PHENOLS.*

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Since Baumann's epoch-making paper on urinary phenol, indican, and the ethereal sulfates (1), considerable work has been done on the quantitative interrelations of these substances, and on the effect of diet on their excretion. Results have been obtained as contradictory as the number of the investigators so that, in these aspects, the literature since Baumann is a dreary desert of mirages. And the reason is not hard to find. In the first place, Folin and Denis (2) have shown that ethereal sulfates must necessarily represent a varying amount of phenol, since the degree of conjugation of these substances is at times quite variable. Furthermore, the methods for phenol determination used by the earlier workers were so crude that only a small part of the urinary phenols could have been accounted for. The phenols, after concentration in alkaline solution and distillation from acid solution, were determined either gravimetrically (3), by precipitation with bromine; or volumetrically (4), by titration with iodine. The latest modification of these methods was proposed in 1912 (5). It still involved prolonged heating with alkali which destroys phenols, as Folin has shown. It is evident that clearer knowledge had to wait on better analytical methods and that, the method at hand, the time is ripe for a study of the effect of diet on the excretion of these substances.

The pioneer work of Herter and Kendall (6) and, more recently, of Rettger and his coworkers (7–9), and of Torrey (10, 11) shows

* The data in this paper are taken from the dissertation presented by George Eric Simpson for the degree of Doctor of Philosophy, Yale University, 1920.

that diet is effective in regulating the character of the intestinal flora. It is, therefore, of prime importance to know the effect of diet on the products of bacterial metabolism eliminated in the urine. Are the changes which are effected in bacterial flora reflected in the quantitative excretion of these products of their metabolism? The most important of these are the aromatic hydroxyacids or phenols, and indican. By putting a patient on a certain regulated diet, can it be said that the processes of bacterial metabolism have thereby been automatically affected, or is it necessary in order to ascertain the efficacy of certain food-stuffs in the regulation of intestinal putrefaction that the bacterial content of the feces be determined? Would the determination of the phenol and indican excretion give the same information?

The present paper gives the results of a quantitative study of the effects of diet on the phenol and indican excretion of the normal individual. By comparison of these results with those of Herter, of Rettger, and of Torrey, we should be able to ascertain how closely excretion of these substances is related to the extent of putrefactive processes in the intestine.

HISTORICAL.

Herter and Kendall (6) determined the effect of diet on the processes of intestinal putrefaction by qualitative bacterial analysis of the fecal flora. When the subjects of their experiments, kittens and monkeys, were on a diet of milk and cane-sugar, the acidophiles rapidly replaced the putrefactive microorganisms which predominated when eggs or meat were the chief constituents of the dietary. They attributed the change to the dominant position of carbohydrate in the milk and cane-sugar diet.

Our present day interest in the effect of diet on the bacteria of the intestinal tract has been crystallized by the work of Rettger and his pupils who have laid especial stress on the influence of lactose in changing the flora in the intestine of rats or of man from predominantly putrefactive to predominantly acidophilic in character. Torrey (10), working on dogs, found that lactose and dextrin were both highly efficacious in transforming the flora of these animals. As little as 30 gm. of lactose or dextrin added to a diet of rice and beef heart were able to produce this change. When starchy foods were fed, there was always a tendency toward the simplification of intestinal flora with elimination of the putrefactive types.

Hirschler (12), a pupil of Baumann's, was the first to demonstrate by *in vitro* experiments the inhibitory action of carbohydrates on intestinal putrefaction. Underhill (13), in feeding experiments with dogs, showed that indican excretion was related to the tryptophane building-stone of

the ingested protein. Homer (14), on the other hand, was unable to find any increased excretion of indican after feeding tryptophane and believed that bacterial action on tryptophane was not a factor in indican formation.

After the development of an accurate method for phenols, Folin and Denis (15–17) were able to carry out far more phenol determinations than had ever previously been reported, and thus provided our first accurate knowledge of the quantitative elimination of these substances. This knowledge of the phenol excretion has been extended by the work of Dubin (18, 19). Moore (20) applied the method to a study ofphenol elimination in infants and found that phenol excretion varied directly with the protein intake, in agreement with Folin's results on the adult.

EXPERIMENTAL.

Since the development of Folin's method for phenols in urine, an extended study of phenol and indican excretion has not been carried out on the normal individual. Such a study is necessary in order that an intelligent interpretation of pathological data may be possible. It has been shown by the results of previous workers that such a study to be of value should meet certain requirements. Any diet should be given for a sufficient time that its effect on the bacterial flora and hence, presumably, on indican and phenol excretion, may become completely established. Since Dubin has shown in his work on dogs that given a uniform diet the phenol excretion tends to remain almost constant in the individual, while the level of elimination may vary markedly in *different* individuals, the effect of various diets should be determined in a single individual in order that comparison may be possible.

For the duration of the experiment artificial stimulation of peristalsis should be excluded, since it would introduce a variable which has been shown to lower the urinary phenol excretion considerably. Furthermore, natural evacuation of the bowel should be regular and complete.

General Plan of the Investigation.

The present paper is based on studies in phenol and indican excretion of a dog extending over a period of 3 months, and of a normal student for a consecutive period of 1½ months at one time and for 15 days at another time. The excretion by two other

normal males was followed for shorter periods. The effect of the following nitrogenous foods, when ingested in large amount, was investigated: gelatin, a tryptophane-free source of nitrogen; meat, a typical animal protein; liver, as an example of glandular tissue high in nuclear material; kidney beans, a vegetable protein presented in the form in which such proteins are usually found in the dietary, that is mixed with a certain amount of indigestible carbohydrate material; and glidine, a mixture of wheat proteins freed from carbohydrate material.

The effect of the carbohydrates, lactose, dextrin (contained in Zwiebach), and starch, was studied in three different individuals. These experiments are not so valuable as they would have been had it been possible to carry them out entirely on the same individual. Nevertheless, they are extremely suggestive.

Methods.

During the course of these experiments, 24 hour urine samples were collected. Since it has been shown by Folin and Denis (17) and by Dubin that little phenol is excreted in the feces compared to the amount in the urine; and by Bergeim (21) that the amount of indole in the feces is exceedingly small, the feces were not collected. On the urines, the determination of total nitrogen, creatinine, phenols, free and combined, and indican was carried out. Total nitrogen was done by the macro Kjeldahl method, creatinine by the method of Folin, indican by Ellinger's permanganate titration method (22), and phenols by the method of Folin and Denis.

All determinations were carried out in duplicate, except the creatinine. When there was a tendency toward variation in the amount of creatinine, duplicate determinations were performed here also.

In the experiments on man it was not found feasible or believed advantageous to regulate the diet completely. At the beginning of the period for urine collection, the experimental diet was taken. This was at noon. For this meal, two rolls were eaten with the experimental nitrogenous mixture. It was found necessary to heat the gelatin and casein with substances to modify their taste. Half the gelatin was mixed with some canned chicken soup and eaten hot. The rest was mixed with a teaspoonful of sugar and

the juice of a lemon and frozen hard. The casein was boiled for some time with stewed tomatoes. A homogeneous, not unpleasant tasting mixture resulted The meat was freed from visible fat, ground, and boiled for ½ hour with a small amount of water. The fat which separated out was poured off, as it was found that otherwise digestive distress ensued. The liver was weighed out and fried. The larger pieces of connective tissue were not eaten. The lactose was mixed with a cup of water and a drop of vanilla extract and taken undissolved.

For supper, Individual A received a small amount of meat (less than ⅛ pound), some potatoes, bread or other food high in carbohydrate, and fruits or vegetables of large cellulose content. For breakfast a roll, prunes, and milk were taken. An endeavor was made to keep the amount of roughage at all times approximately constant. A record was kept of the diet to aid in the interpretation of possible anomalies. During the protein periods the experimental constituents of the diet far overshadowed in quantity, and it is believed in effect on intestinal putrefaction, any other constituents of the diet.

In the experiments on high carbohydrate feeding, carried out on Individuals A, B, and C, the diet contained no meat or legumes. Individual A took 100 gm. of lactose two to four times a day; Individual C, 1¼ pounds of Zwieback to provide a "practical" dextrin diet. The basis of Individual B's diet was rice, bread, potatoes, bananas, and other foods containing a large amount of starch. Individuals A and C supplemented their experimental diet with foods of high starch content.

During the low nitrogen periods, the dog received the following diet:

3.4 gm.	nitrogen as meat, gelatin, casein, kidney beans, etc.
1.1 "	" contained in meat.
110 "	cracker meal containing 2.1 gm. nitrogen.
26 "	lard.
2 "	bone ash.

On the higher level of nitrogen intake, the amount of cracker meal was reduced by half—from 110 to 55 gm.—and the protein intake increased two- to threefold to give the calorific equivalent of the cracker meal.

In the experiments in which 100 gm. of lactose were fed daily in addition to a large amount of meat, the lard was omitted. The diet consisted of:

417 gm. meat.
100 " lactose.
55 " cracker meal.
2 " bone ash.

Lest the presence of fat might be a factor in phenol and indican excretion, during the meat period which followed these lactose-meat periods, it was also omitted, since it was desired to compare the effect of the two diets (see Table IV, D89, 90, and 91).

From the meat most of the fat and connective tissue was removed. It was ground up and preserved on ice in small sterile jars until needed. The beans were prepared by boiling in an autoclave at 15 pounds pressure for 4 hours. The resulting mush was analyzed for nitrogen and substitution was made accordingly. No account was taken of the appreciable proportion of nitrogen which here did not represent protein. The casein was a preparation made 2 or 3 years ago. Cooper's gelatin was used. Merck's milk sugar was used in the lactose experiments. A mixture of wheat proteins was supplied by glidine, a proprietary preparation put up by Menley and James, New York.

Experiments on Man.

Dubin, in his work on the dog, found that when the animal had been on an unvarying diet for a few days the excretion of phenols showed a high degree of constancy, the extreme variations falling under 10 mg. a day. In our dog experiments, phenol excretion attained the same degree of constancy in those cases where the animal was kept for several days on a diet in which meat, casein, or beans were fed (Table III, D1–41).

Our experiments on the human being have not shown this constancy to be characteristic of the subjects of these experiments. Our diets were not so strictly regulated as those of the dogs in Dubin's experiments, especially as to water intake, since it was not found feasible to regulate this carefully.

Then too, the effect of diuresis explains many of the variations in phenol excretion, especially those unexpected high values

obtained sometimes, which are not related to diet changes. Thus, Individual C (Table II, C5–8), while on a high carbohydrate diet, had been excreting an amount of phenol within the range of excretion of the other subjects on similar diets. However, on January 6, the excretion markedly increased—from 295 to 522 mg. of phenol in 24 hours. This increase is associated with, and is to be explained by, the sharp increase in the volume of the urinary output. Thus, in the days when the urine volume was low (C2–4), an average of 740 cc. being excreted daily, the phenol excretion was also low, averaging 342 mg. daily, as compared with that of the 3 days when the urine volume averaged 1,158 cc. and the phenol excretion increased to an average of 530 mg. daily (C5–7).

There is still another factor. In man, certain inhibitions may interfere with the time, and hence probably with the completeness of the evacuation of the bowel, also. In the case of the dog, however, this is not a factor.

That the effect of change of diet on the formation of phenols is completely overshadowed by the factor of bowel evacuation is shown by the high phenol values when the regularity of defecation is interfered with. Although on the previous day there had been a sweeping out of phenol, associated with diuresis, Subject A, on August 5 (Table I, A8), eliminated a much larger amount of phenol, due in part to diuresis, in part, undoubtedly, to failure in evacuation of the bowel. This effect is also seen on August 15, when defecation occurred three times within 24 hours and phenol excretion decreased. But the effect is seen most clearly when the subject was on a meat diet and frequency of defecation increased. The decreased phenol and indican excretion on August 24 and on September 15 is due to no other cause. Thus in days A27 and A39, there was a decrease of over 100 mg. in the amount of phenol eliminated that is not associated either with diuresis or diet changes. On January 1 the effect is even more marked, where, as the result of enhanced evacuation, the urinary excretion of phenol and indican both fell, phenol from 410 to 147 mg. and indican from 23 to 15 mg.

TABLE I.

Indican and Phenol Excretion in Man: Nitrogenous Food-Stuffs.

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P:N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
	1919		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		*mg.*
A1	July 29	Unregulated diet;	795	10.7	1.11	425	216	209	51	49	3.9	3.9
A2	" 30	preliminary.	1,145	12.7	1.27	376	240	136	64	36	3.0	11.0
A3	" 31		1,490	10.3	1.47	506	314	192	62	38	5.1	12.7
A4	Aug. 1		1,200	12.8	1.32	440	231	209	53	47	3.4	10.8
A5	" 2	50 gm. gelatin.	1,110	9.8	1.37	432	262	170	60	40	4.4	6.9
A6	" 3	100 " "	1,670	19.7	1.54	461	278	183	60	40	4.8	10.4
A7	" 4		2,080	20.2	1.54	555	256	299	46	54	2.8	Trace.
A8	" 5	0*	1,890	21.6	1.57	600	331	269	55	45	2.5	"
A9	" 6		2,165	22.3	1.34	525	300	225	57	43	2.3	"
A10	" 7	100 gm. casein.	1,700	20.3	1.56	676	435	241	64	36	3.3	"
A11	" 8		1,510	19.0	1.51	527	349	178	66	34	2.7	11.0
A12	" 9		1,073	19.1	1.47	429	248	181	58	42	2.2	7.3
A13	" 10	100 gm. gelatin. 0*	995	17.8	1.55	478	276	202	58	42	2.7	8.5
A14	" 11		1,300	18.4	1.47	349	214	180	49	51	1.9	Trace.
A15	" 12		1,073	22.9	1.54	471	245	226	52	48	2.2	"
A16	" 13		1,580	19.4	1.10	527	264	263	50	50	2.7	"
A17	" 14		2,060	21.8	1.48	548	278	270	51	49	2.5	"

* The number indicates defecations when this was different from one daily.

A18	Aug. 15	100 gm. casein.	3*	1,415	19.1	1.52	498	259	239	52	48	2.6	1.6
A19	" 16		0*	1,520	18.8	1.38	437	254	183	58	42	2.3	5.6
A20	" 17			1,000	17.3	1.39	417	227	190	54	46	2.4	9.6
A21	" 18			1,420	19.9	1.42	455	257	198	57	43	2.3	10.5
A22	" 19	500 gm. meat.		1,715	19.1	1.57	513	274	239	53	47	2.7	20.4
A23	" 20			1,908	13.4	1.73	525	308	217	59	41	3.9	11.9
A24	" 21	Epistaxis.		1,308	20.8	1.52	661	473	188	72	28	3.2	8.2
A25	" 22	"		1,470	22.2	1.60	521	286	235	55	45	2.3	21.7
A26	" 23	"	4*	1,210	20.4	1.73	466	240	220	52	48	2.3	20.5
A27	" 24	"	2*	920	14.1	1.58	348	179	169	51	49	2.5	6.7
A28	" 25	Influenza.		1,307	11.2	1.79	388	208	180	54	46	3.5	20.0
A29	Sept. 5	100 gm. casein, 100		1,140	17.8	1.52	570	257	313	45	55	3.2	5.8
A30	" 6	gm. lactose.		1,240	22.4	1.70	434	261	173	60	40	1.9	9.2
A31	" 7			966	19.3	2.24	532	260	272	49	51	2.8	11.4
A32	" 8	100 gm. casein.		860	17.0	1.51	430	220	210	51	49	2.5	7.8
A33	" 9			1,155	21.2	1.58	421	226	195	54	46	2.0	6.5
A34	" 10			1,410	18.4	1.65	473	263	210	56	44	2.6	9.2
A35	" 11			1,450	21.0	1.59	473	321	152	68	32	2.3	4.5
A36	" 12	500 gm. meat, 100		1,555	21.7	1.71	430	238	192	55	45	2.0	11.5
A37	" 13	gm. lactose.		1,220	18.7	1.72	406	218	188	54	46	2.2	15.9
A38	" 14			1,000	22.0	1.70	486	245	241	51	49	2.2	15.9
A39	" 15		2*	1,250	21.6	1.70	380	224	156	59	41	1.8	19.0

TABLE I—*Concluded.*

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P:N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
	1919		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		
A40	Sept. 16	500 gm. meat.	1,773	22.5	1.84	531	297	234	56	44	2.4	10.1
A41	" 17	Epistaxis.	1,400	19.7	1.71	432	243	189	56	44	2.2	10.7
A42	" 18	"	1,520	21.7	1.90	447	244	203	55	45	2.1	19.6
A43	" 19	"	1,170	20.5	1.77	421	243	178	58	42	2.0	13.3
A44	Dec. 29	Regular diet.	1,270	10.3	1.87	278	269	9	97	3	2.7	18.0
A45	" 30		957	10.0	1.84	172	148	24	86	14	1.7	17.0
A46	" 31	500 gm. liver.	1,650	18.3	2.03	410	315	95	77	23	2.2	23.0
	1920											
A47	Jan. 1	2*	835	18.5	1.70	147	109	38	74	26	0.8	15.0
A48	" 2		1,040	17.5	1.81	295	214	81	73	27	1.7	20.0

Effect of Diet on Phenol and Indican Excretion.

General.—In the periods when proteins were a preponderating constituent of the diet of Subject A (A1–43) the elimination of phenol is well over 400 mg. a day. The highest value for the daily excretion of total phenols, obtained when defecation frequency was not an interfering factor, was 676 mg. (A10), the lowest 349 mg. (A14), the average, 476 mg.

In the low protein periods, where large amounts of lactose were ingested, however, the phenol excretion was always well under 400 mg. An average of 311 mg. a day was excreted during the 8 days of this régime. Curiously enough, this decrease in phenol excretion on the low protein, high lactose diet is not associated with a decrease in indican excretion. In fact, when we come to consider the experiments on Subjects B and C as well as A, it is seen that indican and phenol excretion may vary quite independently of one another. Thus, Individual B, on a diet containing much carbohydrate, excreted much more indican than Individual A, even on a meat diet, although this diet gave a much higher indican excretion than the lactose diet, if we compare the results on Individual A *inter se*. Nevertheless, the phenol excretion of Individuals B and of A on the carbohydrate diets was of the same degree. Quite different is the case with Subject C. In only one instance did this individual excrete detectable amounts of indican. This individual was excreting far more phenols than either Subjects B or A, although all were on a diet containing large amounts of carbohydrate. Phenol and indican excretion, then, in different individuals, may bear quite a different relation to one another. May this be associated with differences in the character of the flora—certain strains being especially prone to metabolize tryptophane, for instance?

Consideration of the ratio of the total phenols to total nitrogen should answer the question: Does total phenol excretion parallel the total nitrogen of the food? When there is incomplete absorption of the food, shown by a low nitrogen excretion, is there a tendency toward the manufacture of indican and phenol in larger amounts than usual? In the experiments on man it is seen that this ratio tends to become constant when the diet is regulated, and that the highest values are found during the high

TABLE II.

Indican and Phenol Excretion in Man: Carbohydrate Diet.

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P:N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
Subject A: Lactose.												
	1920		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		*mg.*
A49	Jan. 3	200 gm. lactose.	990	9.1	2.10	249	200	49	80	20	2.7	10
A50	" 4	200 " "	980	6.9	2.02	297	175	122	59	41	4.3	12
A51	" 5	400 " " *	1,370	6.4	2.04	311	182	129	58	42	4.9	8
A52	" 6		1,440	5.8	1.96	327	185	142	56	44	5.7	6
A53	" 7		1,055	5.3	1.99	377	203	174	54	46	7.1	11
A54	" 8		1,200	5.6	1.78	314	177	137	56	44	5.6	4
A55	" 9											
A56	" 10		580	4.5	1.77	268	132	136	49	51	5.9	4
A57	" 11		1,335	6.4	1.94	338	236	102	70	30	5.3	9
A58	" 12		760	5.9	1.52	260	170	90	65	35	4.4	11
Subject B: Starch.												
B1	Jan. 2	Regular diet.	850	9.1	1.98	424	228	196	54	46	4.7	7
B2	" 3	Starch " *	1,590	10.6	2.02	310	239	71	77	23	2.9	27
B3	" 4		1,120	8.9	1.81	307	193	114	63	37	3.5	34
B4	" 5		1,325	10.3	1.92	306	195	111	64	36	3.0	32
B5	" 6		1,030	8.8	1.70	262	153	109	58	42	3.0	23
B6	" 7		830	9.8	1.77	392	184	208	47	53	4.0	30

* This diet continued for the duration of the experiment.

B7	Jan. 8		1,140	10.9	1.73	373	185	188	50	50	3.4	17
B8	" 9		870	10.3	1.83	353	203	150	58	42	3.4	34
B9	" 10		1,520		2.02	387	225	162	58	42		23
B10	" 11		1,080	9.5	1.97	378	241	137	64	36	3.9	14
B11	" 12		1,145	9.2	1.63	394	263	131	67	33	4.3	10
B12	" 13		660	9.6	1.46	389	258	131	66	34	4.0	17
		Subject C: Dextrin.										
C1	Jan. 2	Regular diet.	825	8.2	1.90	393	169	224	43	57	4.8	None.
C2	" 3	Dextrin " †	880	8.2	1.93	383	245	138	64	36	4.7	"
C3	" 4		690	9.3	2.70	349	216	133	62	38	3.7	"
C4	" 5		650	9.9	2.20	295	116	179	39	61	3.0	"
C5	" 6		1,350	9.1	1.67	522	318	204	61	39	5.7	"
C6	" 7		1,230	8.2	1.84	550	301	249	55	45	6.6	"
C7	" 8		895	11.7	1.79	516	162	344	33	67	4.4	"
C8	" 9											
C9	" 10		890	11.7	1.72	450	247	203	55	45	3.8	None.
C10	" 11											
C11	" 12		1,130	7.4	2.08	464	354	110	76	24	6.2	16.0
C12	" 13		900	8.0	1.66	417	292	125	70	30	5.2	None.

† The diet contained approximately 1½ pounds of Zwiebach for the rest of the experimental period.

lactose period. Is this, perhaps, due to the stimulating effect of this relatively insoluble carbohydrate on bacterial metabolism so that, from the small amount of nitrogen available, a comparatively large amount is utilized by the microorganisms? It cannot be overlooked that in this case the small amount of protein was supplied by potatoes and bread. The coefficient of digestibility of these proteins has been shown by Mendel and Fine (23) to be the same as that of meat. In the form of bread, however, the protein is evidently protected from the action of the digestive juices since the nitrogen is less available than that of meat, as shown by Woods and Merrill (24). In this case, therefore, more undigested residue may reach the sphere of action of the bacteria in spite of the low nitrogen intake, and thereby give rise to an increased excretion of indican and phenol. Of the two influences, it cannot be determined which was effective.

In the period during which the higher level of nitrogen intake was maintained, the conjugated phenols represented between 40 and 50 per cent of the total phenols. In only four instances did the degree of conjugation fall below this limit (Table I, A10, 11, 24, and 35). In the first and third, the decrease was associated with a sudden increase in total phenols, with the actual amount of conjugated phenols remaining at the same level. In general, it may be said that the degree of conjugation remained the same in spite of the wide differences in nitrogen intake. The extent of conjugation rose and fell with the total phenol excretion except when sudden variations were encountered. The stimulating effect of high phenol excretion on the degree of conjugation, noted by Dubin, is not apparent from our experiments, except that the actual amount of conjugated phenols increased enough to keep the proportion of conjugated phenols the same.

Gelatin.—When gelatin was the chief source of nitrogenous food (Table I, A5–9, 13–17), there was always, within a day or two, a fall to a mere trace of indican in the urine. This reappeared as soon as the gelatin was replaced by casein. The disappearance in this case is not associated with any marked change in the phenol output. The swiftness of the disappearance and reappearance gives an idea of the time necessary for our experimental nitrogenous food-stuffs to exert their effect in predominating the metabolism of the intestinal flora. In studying the

effect of casein, meat, and the meat-lactose combinations, it will be seen that phenol and indican excretion always showed a tendency to vary in the same direction in this individual. That they do not do so in this instance is merely an expression of the fact that gelatin is lacking in the building-stone which gives rise to the indican. Thus our results confirm those of Underhill, who determined the indican excretion of dogs when gelatin was fed as the predominating source of nitrogen. It was found that there was an invariable decrease on this diet, as compared with meat as the nitrogenous food.

Casein.—When casein was the chief source of nitrogen, phenol excretion was at the same level as with gelatin, while the indican excretion increased and attained a level usually somewhat under 10 mg. of indigo in 24 hours (Table I, A10–12, 18–21, 32–35). In the second period of casein feeding the casein influence was established immediately and 1.6 mg. of indigo were found in the urine collected 24 hours after its feeding had begun. This quick establishment was due to the fact that the debris from the preceding gelatin period had been removed from the sphere of action of the bacteria as the result of the increased frequency of defecation. The slight diarrhea was probably due to mechanical irritation of the walls of the intestine by particles of casein. These were sharp cornered because the casein had not been sufficiently softened by boiling before it was eaten.

Meat.—When a diet containing large quantities of meat followed a diet of casein there was always a sharp increase in the amount of indican in the urine (Table I, A22–28, 36–43). This increase appeared the 1st day the meat was fed and soon resulted in the excretion of about 20 mg. of indigo a day. During the meat period the phenol values, as well, tended to become higher than in the preceding casein régime. While this effect is not seen so clearly in the second series of meat experiments, it is invariably seen in the experiments we have carried out on the dog. During the period of meat feeding the indican values were quite variable —much more variable than on the casein diet. This diet in both cases gave rise to increased frequency of defecation. This resulted in sharp decreases in the excretion of both phenols and indican.

In both instances the meat diet gave rise to very disagreeable subjective symptoms. It caused epistaxis, as well as decreased mental and physical efficiency. During the first series of experiments on this diet, a midsummer cold developed. During the second series, symptoms of a cold were appearing, as well as the epistaxis, so that the experiment was discontinued. In view of these results the diarrhea produced on these meat diets seems to the writer to be a protective mechanism to aid in getting rid of harmful and poisonous materials.

Why should meat differ from gelatin and casein in causing the relatively high excretion of phenol and indican? There are two differences between meat and the other nitrogenous substances which may have contributed to this effect. The meat was always mixed with a certain amount of fat, which may have caused more of the protein material to pass to the lower gut undigested. The larger part of the fat was always drained off, however, and, as no butter was eaten during this period, the total amount of fat eaten was not greater than on the other diets. In the second place meat is unique among our experimental foods in having a certain mixture of indigestible protein, the connective tissue protein. Therefore, a greater amount of material containing tryptophane and tyrosine building-stones would reach the field of bacterial activity and enter into bacterial metabolism.

Liver.—The ingestion of 1 pound of liver a day (Table I, A46–48) brought about an immediate increase in the phenol and indican excretion. Although the clearness of the result was partly obscured by the very complete evacuation of the bowel on the 2nd day, it is evident that liver, rich in nuclear material, does not differ from muscle tissue in its effect on intestinal putrefaction.

Carbohydrate Foods.—The effect of the addition of small amounts of lactose to a diet high in protein was studied in order to determine if this would have an inhibitory effect on bacterial metabolism. The object of these experiments was to have present in the diet an amount of lactose which might be ingested in the form of milk. No change was seen during the few days in which this diet was fed, either in phenol or in indican excretion (Table I, A29–31, 36–39). In a course of milk feeding which lasted 5 days, 4 quarts of milk daily being ingested, no decrease

in phenol or indican excretion was seen. The value for both phenol and indican was intermediate between our casein and meat values. Constipation was an interfering factor in these experiments, which could not be avoided. Therefore, these results have not been included in this report.

In a second series of experiments with carbohydrates, three subjects were kept for 8 to 10 days on a régime in which carbohydrate was supplied in much larger quantities. Rettger has shown that lactose changes the bacterial flora in the course of a few days from predominantly putrefactive to predominantly acidophilic. Torrey found that in puppies the addition of bread to a milk diet caused a considerable change in the flora of the intestine. He attributed the consequent replacement of *Bacillus coli* by *Bacillus acidophilus* to the dextrin content of the bread, in part. It seemed of interest, then, to perform experiments which should be comparative and thus determine the effect of these carbohydrates on the excretion of phenols and indican. Unfortunately, our results are not comparable *inter se* on this point since it was not possible to carry them all out on the same individual, and our original question has not been answered.

The excretion of indican and phenol by Subjects B and C did not differ materially from the excretion during the preliminary period. The results lead to one definite conclusion and that is that, by putting a subject on a diet of high carbohydrate content —a "practical" diet—whether the carbohydrate is lactose, or dextrin from toast, or starch, it cannot from that alone be said that the excretion of the products of intestinal putrefaction has ceased, or even come to a low level. In order to know what has happened as the result of such treatment it is indispensable either that studies of fecal bacteria be carried out, or that the excretion of phenol and indican be followed in a quantitative way. Thus, Subject C, on dextrin diet, on only one occasion excreted indican in the urine; Subject A, on lactose, excreted a small amount of indican; while Subject B, on starch, excreted very large amounts of indican compared with the others. The phenol excretion of all subjects was low as compared with the values obtained on Subject A on high protein diets. The high phenol values found on Subject C (C5–7) are a result of the diuresis which followed days of small urinary output.

In respect to the experiments on lactose, these may be compared with the other experiments on Subject A. On this diet the nitrogen output was low. The phenol excretion was much lower than on the high nitrogen diets. The indican also was low. The beginning of the period of lactose feeding was accompanied by the development of large amounts of gas in the alimentary tract with very marked distention of the abdominal wall. This lasted until the 7th day, when the distress was considerably diminished, while on the 8th and succeeding days there was no notable development of gas. This gas production we have interpreted as due to the predominance of the putrefactive organisms which are able to convert lactose into carbon dioxide, and the decrease in the amount of gas we have interpreted as due to the displacement of the putrefactive organisms by the acidophiles. It would have been of great interest to continue these experiments a few days longer. Unfortunately, however, it was necessary to discontinue them so that it remains for future experiments to show whether the effect which Rettger and his coworkers have found for lactose in reducing putrefactive bacteria would have been associated with a further decrease in the excretion of urinary phenols and indican.

Experiments on the Dog.

The experiments on the dog may be divided into two groups. In the first (Table III, D1-41), the nitrogen intake was comparatively low. The diet was regulated to give about 0.9 gm. of protein per kilo. The nitrogen excretion on this régime was about 5 gm. a day, except on the bean diet where absorption was impaired. In the second group of experiments the amount of carbohydrate was reduced while the protein intake was increased so that the same energy value was provided. The cracker meal allotment was reduced by half and the protein intake increased approximately threefold to make up this difference. The nitrogen excretion was twice, and sometimes threefold that on the low nitrogen diets.

For 2 weeks previous to the experimental periods, the animal had been kept on a diet of meat scraps and cracker meal. For some time after the experimental period there was a tendency

for the phenol and indican values to vary considerably. The general trend was toward smaller values irrespective of the diet. Thus, during the first 34 days of the experiments the phenol excretion decreased, whether the nitrogenous food was meat (D1–8), gelatin (D9–18), casein (D19–26), or again meat (D27–34). This decrease is undoubtedly the expression of the simplification of the bacterial flora of the intestinal tract, noted by Herter and Kendall in their experiments on kittens and monkeys.

The phenols and indican in these first experiments do not seem to be affected by changes in the type of protein in the food, except that after the indican had disappeared on a casein diet, traces again appeared in the urine when meat replaced the casein. In the second period of meat feeding, nitrogen excretion was distinctly smaller than in the first meat period, while the phenol to nitrogen ratio was larger than at any other time on this type of diet. Unfortunately it was not noticed whether this meat was especially tough or contained an unusual amount of indigestible connective tissue. This seems, however, the plausible explanation for the effect noted.

When beans were fed as the source of nitrogen, the indican again disappeared. The phenols underwent a small but clearly defined increase. This increase in phenol excretion was associated with a decided fall in the excreted nitrogen, although the amount of nitrogen in the food was the same as on the preceding meat diet. The decrease in nitrogen elimination means that an appreciable quantity of bean nitrogen was not being absorbed from the alimentary tract. With the increase in phenols and the decrease in total nitrogen there was a decided increase in the ratio of phenol to total nitrogen, so that it averaged nearly three instead of less than two, as on all other low nitrogen diets.

There was a distinct and immediate rise in phenol excretion when the nitrogen intake was increased two to three times. The ratio of total phenol to total nitrogen, however, remained at the same level as on the low nitrogen diets.

Gelatin.—From July 23 to August 5 the dog was kept on a diet in which gelatin was the source of nitrogen. It was impossible to induce the animal to take this diet with any degree of consistency and the attempt was finally abandoned. The daily

TABLE III.

Indican and Phenol Excretion in the Dog: Low Protein Diets.

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P : N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
	1919		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		*mg.*
D1	May 14	136 gm. meat.	432	5.1	0.24	91	60	31	66	34	1.8	1.8
D2	" 15		286	5.3	0.24	84	34	50	65	35	1.6	1.6
D3	" 16		306	5.4	0.18	104	64	40	63	37	1.9	2.3
D4	" 17		402	5.6	0.26	111	66	45	60	40	2.0	2.9
D5	" 18		316	5.3	0.24	82	53	29	65	35	1.5	1.4
D6	" 19		330	5.7	0.23	87	56	31	64	36	1.5	2.6
D7	" 20		325		0.23	89	50	39	55	45		1.5
D8	" 21		286	5.6	0.23	93	55	38	59	41	1.6	1.3
D9	" 22	18.7 gm. gelatin.	286	5.9	0.22	95	53	42	56	44	1.6	0.8
D10	" 23		250	5.6	0.29	94	64	30	68	32	1.7	1.5
D11	" 24		292	5.2	0.22	120	65	55	54	46	2.3	1.3
D12	" 25		234	5.2	0.21	86	48	37	57	43	1.7	0.1
D13	" 26		325	5.1	0.18	83	48	35	58	42	1.6	0.7
D14	" 27		272	5.3	0.23	75	44	31	59	41	1.4	2.2
D15	" 28		360	5.3	0.22	81	42	39	53	47	0.5	3.1
D16	" 29		248	5.0	0.23	79	48	31	61	39	1.6	0.5
D17	" 30		270	4.6	0.23	67	36	31	55	45	1.4	1.6
D18	" 31		252	4.6	0.22	71	38	33	54	46	1.5	3.3

D19	June	3	27 gm. casein.	248	4.6	0.21	84	54	30	64	36	1.8	0.8
D20	"	4		246	5.0	0.21	89	55	34	62	38	1.8	2.3
D21	"	5		149	3.8	0.23	86	50	36	59	41	2.2	None.
D22	"	6		200	5.0	0.20	85	56	29	66	34	1.7	"
D23	"	7		309	5.1	0.20	75	48	27	64	36	1.5	"
D24	"	8		238	4.7	0.22	75	46	29	61	39	1.6	"
D25	"	9		326	4.0	0.19	76	45	31	59	41	1.9	"
D26	"	10		380	4.3	0.10	74	50	24	67	33	1.7	"
D27	July	1	136 gm. meat.	312	3.3	0.20	65	53	12	82	18	2.0	1.0
D28	"	2		345	3.6	0.24	59	41	18	69	31	1.6	Trace.
D29	"	3		(?)	3.2	0.24	78	54	23	71	29	2.4	"
D30	"	4		228	3.4	0.24	84	61	23	71	29	2.5	"
D31	"	5		218	3.5	0.20	79	56	23	72	28	2.3	"
D32	"	6		412	3.6	0.22	84	62	22	75	25	2.3	"
D33	"	7		328	3.8	0.23	82	60	22	73	27	2.2	"
D34	"	8		282	4.0	0.25	71	55	16	77	23	1.8	"
D35	"	9	94 gm. beans.	166	3.1	0.23	88	59	29	67	33	2.8	None.
D36	"	10		176	3.1	0.23	88	52	37	58	42	2.8	"
D37	"	11		316	2.9	0.22	83	60	23	72	28	2.9	"
D38	"	12		270	2.8	0.23	85	52	23	71	39	3.0	"
D39	"	13		200	2.6	0.22	73	48	24	77	33	2.8	"
D40	"	14		270	2.8	0.24	82	42	40	51	49	3.0	"
D41	"	15		254	3.9	0.24	94	64	30	78	32	2.4	"

nitrogen elimination during this time ranged from 1.8 to 10.2 gm. The animal alternately fasted, or ate the gelatin. The two effects were hopelessly confused and these experiments are not reported in the tables.

Casein and Meat.—The experiments on meat feeding and on casein feeding confirm the results obtained with man.

The period of casein feeding was of longer duration than in our human experiments so that its effect has become more clearly established. The conclusion was the same, however. Indican and phenol were both excreted in appreciable amounts on this diet. The phenol as well as the indican output per day was distinctly higher on the meat diet. The effect of the meat diet on the physical condition of the animal was very marked. While on all other diets the animal was very lively and seemed contented, during the periods of meat feeding she became morose, stupid, and slept a large part of the time. When awake she kept chewing at the bars of the cage. After a few days the feces became black in color and tarry in consistency. By the 3rd or 4th day the animal either refused the food or, after eating, vomited. The diarrhea was undoubtedly a mechanism adapted to ridding the system of toxic materials.

Vegetable Proteins.—When glidine was fed in large amounts (Table IV, D67–71), the phenol and indican excretion attained about the same level as in the casein experiments. Indican experienced a sudden drop from 20 mg., on the last day of the preceding meat diet, to 5 mg. on the first glidine day. After this the amount of indican slowly fell to 1 mg. on the 5th and last day of the experiment. Unfortunately the animal refused to eat this diet any longer and subsequent attempts to carry out an experiment on this diet were not successful. We cannot, therefore, be certain whether indican excretion might not have ceased entirely. However, on all other diets, the level of indican excretion established on the 4th day was maintained throughout the period and there is no reason to believe the wheat proteins would have been an exception to this.

In contrast to the other vegetable protein, the bean protein which had been fed during the low nitrogen experiments, the wheat protein was fairly well absorbed as indicated by the urinary nitrogen. This is in agreement with the results of Mendel and

Fine (23). The ratio of total phenol to total nitrogen was at the usual level. In this case, then, there was no unusual amount of protein reaching the lower gut and causing enhanced activity of the putrefactive organisms, which would be followed by increased phenol excretion. This difference in the two vegetable proteins was merely the result of the fact that the bean protein was to a certain extent encased in an envelope of difficultly digestible material which prevented complete absorption. The wheat protein had been freed from this indigestible carbohydrate.

Carbohydrates.—Attempts were made to determine the effect of the addition of large amounts of carbohydrate to the high meat diets. In one experiment cracker meal was used, in the other, lactose. When the cracker meal and meat mixture was fed after a glidine period, there was a definite increase in phenol excretion and also in indican excretion. The amount of these substances in the urine was not so large as was usually found in the urine on a meat diet, although the diet was fed for 5 days.

The lactose experiment was of shorter duration than would permit the drawing of clear-cut conclusions. As the result of the preceding 6 days fasting, phenol and indican had reached very low levels. The metabolism of the putrefactive organisms had decreased by reason of the small amount of metabolite. As soon as the meat and lactose diet was fed (Table IV, D86–88), the phenol and indican in the urine both increased. In order for the effect of the lactose in possible transformation of the flora to become evident a longer period of lactose feeding would obviously have been necessary.

When the meat and lactose diet was replaced by a diet containing meat alone (D89–91), there was an immediate sharp increase in both indican and phenol excretion. This would indicate that there may have been an inhibitory action from the lactose, due to the favoring of acidophilic organisms. However, when we compare the excretion of phenol and indican on the lactose period with the excretion of these substances during the preceding meat period (Table IV, D80–82), we see that there is really no distinct difference. Our results on carbohydrate feeding are suggestive rather than conclusive.

TABLE IV.

Indican and Phenol Excretion in the Dog: High Protein Diet; The Effect of Fasting.

Sample No.	Date.	Remarks.	Volume.	Total N.	Creatinine.	Phenols.					P : N ratio.	Indican as indigo.
						Total.	Free.	Conjugated.	Free.	Conjugated.		
	1919		*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*		*mg.*
D42	July 16	82 gm. casein.	365	3.4	0.24	97	69	71	71	29	2.9	None.
D43	" 17		410	8.9	0.26	160	113	47	71	29	1.8	"
D44	" 18		410	10.0	0.26	140	93	47	66	34	1.4	1.9
D45	" 19		355	8.6	0.28	116	85	31	74	26	1.3	1.5
D46	" 20		410	9.9	0.24	152	104	48	69	31	1.5	2.1
D47	" 21		262	8.0	0.24	86	60	26	70	30	1.1	2.6
D48	" 22		275	8.5	0.25	91	62	29	68	32	1.1	3.3
D49	" 23	79 gm. gelatin.	650	10.3	0.21	86	37	49	57	43	0.8	4.2
D63	Aug. 6	417 " meat.	425	10.1	0.56	174	109	65	62	38	1.7	12.2
D64	" 7		315	9.4	1.25	302	178	124	59	41	3.2	53.2
D65	" 8		414	14.4	1.14	295	165	130	56	44	2.0	65.4
D66	" 9	Feces diarrheal.	282	9.8	0.91	164	101	63	62	38	1.7	19.8
D67	" 10	87.5 gm. glidine.	236	9.6	0.40	129	81	48	63	37	1.3	5.1
D68	" 11		220	12.6	0.41	134	89	45	67	33	1.1	4.2
D69	" 12		325	13.8	0.43	117	109	8	93	7	1.8	6.0
D70	" 13		335	12.1	0.38	141	95	46	67	33	1.2	2.3
D71	" 14	All food not taken.	214	7.2	0.43	133	95	38	71	29	1.8	1.0
D72	" 15	417 gm. meat, 165	356	12.4	0.81	172	127	45	74	26	1.4	5.8
D73	" 16	gm. cracker meal.	380	12.5	1.00	185	136	49	74	26	1.5	7.4

D74	Aug.	17		487	11.4	0.96	159	119	40	75	25	1.4	5.8
D75	"	18		250	22.0	0.78	163	115	47	71	29	0.7	3.0
D76	"	19	Vomits.	280	17.5	1.00	175	136	39	78	22	1.0	5.7
D77	"	20	Fasting.	164	4.9	0.44	107	76	31	71	29	2.2	6.5
D78	"	21		86	4.2	0.27	72	51	21	71	29	1.7	2.5
D79	"	22		77	2.7	0.28	73	53	21	73	27	2.7	2.7
D80	"	23	417 gm. meat.	202	7.9	0.86	126	100	26	79	21	1.6	5.5
D81	"	24		355	10.4	1.14	133	98	35	74	26	1.3	10.7
D82	"	25		540	9.0	0.98	132	94	38	71	29	1.5	12.2
D83	Sept.	2	Fasted since Aug. 29.	35	1.7	0.24	37	26	11	70	30	2.1	0.8
D84	"	4		45	2.2	0.24	39	30	9	77	23	1.8	None.
D86	"	6	417 gm. meat, 96 gm.	510	11.8	0.98	204	155	49	76	24	1.7	2.4
D87	"	7	lactose.	375	10.0	0.79	181	128	53	71	29	1.8	5.7
D88	"	8	One-half food eaten.	320	8.6	0.72	165	112	53	68	32	1.9	19.9
D89	"	9	417 gm. meat.	310	3.8	0.95	248	147	101	59	41	6.5	35.9
D90	"	10		570	12.1	0.87	226	140	86	62	38	1.9	40.8
D91	"	11		282	14.3	0.71	266	161	105	61	39	1.8	31.7
D92	"	12	Fasting.	89	3.7	1.55	86	48	37	57	43	2.3	4.0
D93	"	13		72	3.2	0.28	61	50	11	82	18	1.9	2.7
D94	"	14		404	3.5	0.24	129	98	31	76	24	3.7	0.7

Fasting.—The results of inanition as seen in our experiments are in agreement with those of Dubin. There is a sharp decrease in the excretion of phenol, indican, and total nitrogen. The ratio of total phenol to total nitrogen does not change.

DISCUSSION.

When the results of Herter and Wakeman, of Rettger and his pupils, and of Torrey, who determined the effect of diet on the bacterial flora of the feces, are compared with our results on the effect of diet on the excretion of indican and the phenols, the relation between the two is established. Those diets which gave rise to the excretion of phenols and indican in large amounts were those diets which promoted the growth of the putrefactive microorganisms in the intestine. Thus a diet containing large amounts of meat was found, by Herter and Wakeman, and by Torrey, to promote the growth of the putrefactive bacteria. Such a diet greatly stimulates the excretion of indican. The excretion of the phenols is also increased on this diet. The effect is not seen so clearly in man as in the dog, because in man there are wider variations in urine flow and the completeness of the emptying of the bowel.

Rettger and Torrey have shown that lactose is very efficacious in bringing about a predominance of the acidophiles. Torrey found that when starchy foods were fed there was a tendency toward elimination of the putrefactive types of bacteria. White bread and potatoes had this influence. On this type of diet, we have found that the excretion of phenols, especially, is lower than on any other type of diet.

Casein was found by Torrey to show a much smaller tendency to give rise to intestinal putrefaction than meat. We have found that this protein causes far less phenol and indican to appear in the urine than the meat diet.

Herter and Kendall found that, in the earlier stages of their experiments on kittens and monkeys, sudden shifts of diet were not accompanied by like changes in the character of the fecal flora. As their experiments were continued, however, sharper variations began to appear. They attributed this effect to the simplification of the flora caused by the elimination of all but

the predominant putrefactive or acidophilic strains. Thus we have here, in the parallelism of our results with those of Herter and Kendall, another indication that variation in urinary indican and the phenols is associated with changes in the character of the intestinal flora.

Torrey found that the "Vegetable proteins stand in strong contrast to animal proteins, especially meat, in that they do not offer the slightest encouragement to the growth of intestinal putrefactive types of bacteria." Hull and Rettger, on the other hand, found that a "Diet of bread and vegetables does not encourage this flora (the acidophiles), but has the same influence as ordinary protein diet." In this instance our results follow those of Hull and Rettger rather than those of Torrey. For we have found that a mixture of wheat proteins was on the same level as the animal protein, casein, as far as the excretion of indican and phenol was concerned, and that the elimination of these substances was much higher on such a diet than when a diet containing a large amount of carbohydrate was fed.

It is very evident from our results that the determination of both indican and the phenols in the urine gives a much better indication of the extent of intestinal putrefaction than the determination of either of these substances alone. The extreme differences in different individuals in their tendency to excrete indican, especially, makes comparisons between individuals, in this respect, hazardous.

SUMMARY.

In a study of the urinary excretion of indican and the phenols, carried out on three normal individuals and on a dog, the following results were obtained:

1. The excretion of both indican and the phenols varies directly with the protein intake. In their smaller fluctuations, the excretion of phenol and indican does not necessarily vary in the same direction. In most cases, a *large* variation in indican excretion is accompanied by a variation in phenol excretion in the same direction.

2. In different individuals the phenol and indican excretion may bear quite a different relation to one another. A high indican excretion by an individual, as compared to other indi-

viduals, does not necessarily mean a correspondingly high excretion of phenols by that individual.

3. The effect of even mild constipation overshadows the effect of diet on the excretion of these substances. Constipation causes a large increase in the excretion of indican and the phenols. Diuresis, also, is shown to promote the excretion of these substances.

4. With increased phenol excretion there is stimulation of conjugation to the extent that in most cases the same *percentage* of the total phenol is excreted in conjugation.

5. Comparison of our results with those of Herter and Kendall, of Rettger and his pupils, and of Torrey indicates that those diets which promote the growth of the putrefactive bacteria also promote indican and phenol excretion.

(*a*) Meat ingested in large quantities causes a marked increase in the excretion of the phenols and indican.

(*b*) Casein causes a moderate excretion of indican and the phenols.

(*c*) Gelatin, containing no tryptophane building-stone, causes cessation of indican excretion with no change in the excretion of the phenols.

(*d*) Glidine, a mixture of wheat proteins, did not differ from casein in its effect.

(*e*) Kidney bean protein, fed with the usual admixture of indigestible carbohydrate substances, was less completely absorbed than the other proteins. Phenol excretion was increased by a small, but definite, amount.

(*f*) On a diet containing large amounts of lactose the excretion of indican and the phenols was lower than when the diet contained a large amount of protein.

6. In intelligent dieting with the regulation of intestinal putrefaction as the aim, it is necessary to study either the fecal flora or the excretion of phenols and indican.

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BIOCHEMICAL STUDIES ON MARINE ORGANISMS.

I. THE OCCURRENCE OF COPPER.

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INTRODUCTION.

The occurrence of copper in tissues of certain marine animals has been known for a long time. More than 70 years ago Harless (1847) detected copper in the blood of *Eledone* and *Helix pomatia*, and showed that it exists not as a free salt, but in combination with protein. This peculiar copper-containing protein, which was subquently named hemocyanin by Frédéricq (1878), is apparently an oxygen carrier analogous to the hemoglobin of higher animals. It differs in locality from the latter in occurring in the plasma rather than in the form elements of the blood. Unlike hemoglobin it cannot be disintegrated into a protein and a protein-free component. When shaken with air, hemocyanin combines loosely with oxygen, and is converted into blue oxyhemocyanin, and by the escape of the oxygen becomes colorless again. According to Henze (1901), hemocyanin of the *Octopus* may be crystallized, and the crystalline preparation has the following composition: C = 53.66; H = 7.33; N = 16.09; S = 0.86; O = 21.67; and Cu = 0.38 per cent.

Abundant evidence has been adduced to prove that hemocyanins of different sources are not identical in composition or physical properties. In a series of investigations Dhéré (1900, 1903, 1913, 1914, 1919) examined the blood of various mollusks and arthropods, and noted peculiarities in crystallization, percentage of copper, and color reactions of the respective hemocyanins. He states that the efficiency of a hemocyanin as an oxygen carrier is directly proportional to its copper content.

This latter observation is of considerable physiological interest. In many cases at least, the more active species requiring a rapid respiratory exchange are provided with blood containing larger percentages of copper than other species of a low metabolic type. This fact was emphasized by Mendel and Bradley (1906–07). Thus the blood of the highly active *Octopus* contains 18 to 23.5 mg. of copper per 100 cc., while the very inactive *Helix pomatia* is provided with only 6.5 to 12.5 mg. per 100 cc. More recently Alsberg and Clark (1910–11) have made a careful investigation of the hemocyanin of *Limulus polyphemus*, and find that it differs materially from the preparation studied by Henze. *Limulus* hemocyanin contains 0.28 per cent of copper, is much richer in sulfur and oxygen, and contans less carbon than the respiratory protein of *Octopus*. The copper in Alsberg and Clark's preparation was in an exceedingly loose combination, which could be completely removed by dialysis if the water used were kept very faintly acid.

One of the most interesting differences in hemocyanins is that noted by Mendel and Bradley (1905, 1906–07) in the case of the marine gastropod, *Sycotypus canaliculatus*. The respiratory protein of this mollusk was found to contain zinc in addition to copper. The name "hemosycotypin" was suggested for this peculiar oxygen carrier. Numerous other investigators, notably Lankester (1872–73), Krukenberg (1880), Halliburton (1885), Griffiths (1890–91, 1892), and Philippi (1919), have studied hemocyanins of various sources. The earlier investigations are discussed in detail in the excellent review of von Fürth (1903). The fact which we wish to emphasize here is the wide occurrence of copper as a constituent of invertebrate blood. Nor is this metal confined to the circulating fluid, but may occur in other portions of the body as well. Through the investigations of Herdman and Boyce (1898–99), Nelson (1915), and others, copper is known to occur in relatively large and variable amounts in oysters. According to Willard (1908), and Hiltner and Wichmann (1919), this metal may be present in sufficient amount to impart a greenish tint to the oysters, or even to produce toxic symptoms when they are ingested by man.

In Table I are listed the animals in which copper is known to occur, either as hemocyanins in the blood, or stored in unknown combinations elsewhere in the organism.

TABLE I.

Animals in Which Copper is Known to Occur.

Animal.	Part analyzed.	Reference.
Echinodermata.		
Asterias.....	Entire animal.	Dubois (1900).
Echinus.....	" "	" (1900).
Stichopus.....	" "	" (1900).
Holothuria.....	" "	Phillips (1917).
Annulata.		
Hirudo.....	" "	Dubois (1900).
Crustacea.		
Palaemon.....	" "	" (1900).
Clibanarius	" "	" (1900).
Astacus.....	Blood.	Griffiths (1890–91).
Squilla.....	"	Krukenberg (1880).
Palinurus.....	" and liver.	Griffiths (1890–91); Phillips (1917).
Nephrops.....	"	Halliburton (1885).
Homarus.....	"	" (1885); Griffiths (1890–91).
Carcinus.....	"	Griffiths (1890–91).
Maja.....	"	Kobert (1903).
Eriphia.....	"	Krukenberg (1880).
Cancer.....	" and liver.	Harless (1847); Griffiths (1890–91).
Arachnida.		
Scorpio.....	"	Lankester (1872–73).
Limulus.....	"	" (1872–73); Alsberg and Clark (1910–11).
"	Liver, ovaries, etc.	Phillips (1917).
Pelecypoda.		
Ostrea.....	Entire animal.	Herdman and Boyce (1898–99). Willard (1908). Hiltner and Wichmann (1919).
Anodonta.....	Blood.	Griffiths (1890–91).
Mytilus.....	"	" (1890–91).
Pinna.....	"	" (1890–91).
Mya.....	"	von Fürth (1903).
Pecten.....	Entire animal.	Dubois (1900).
Unio.....	" "	" (1900).
Gastropoda.		
Helix.....	Blood.	Harless (1847).
Murex.....	"	Kobert (1903).
Turbo.....	"	" (1903).

TABLE I—*Concluded.*

Animal.	Part analyzed.	Reference.
Aplysia	Liver.	Phillips (1917).
Fissurella	Blood.	Kobert (1903).
Haliotis	"	" (1903).
Sycotypus	" and liver.	Mendel and Bradley (1905).
Fulgur	Liver.	Bradley (1904); Phillips (1917).
Cassidaria	Blood.	Kobert (1903).
Fasciolaria	Liver.	Phillips (1917).
Cassis	"	" (1917)
Strombus	Soft parts.	" (1917).
Triton	Blood.	von Fürth (1903).
Arion	"	" " (1903).
Cyclostoma	"	" " (1903).
Scaphander	"	" " (1903).
Paludina	"	" " (1903).
Cephalopoda.		
Loligo	"	Kobert (1903).
Sepia	"	Griffiths (1890–91).
Octopus	"	Frédéricq (1878); Henze (1901).
Eledone	" and liver.	Harless (1847); Dhéré (1913).
Tunicata.		
Ciona		Phillips (1917).
Elasmobranchii.		
Acanthias	Liver.	Harless (1847).
Teleostomi.		
Zeus	"	" (1847).
Conger	"	" (1847).

As will be observed, the data thus far accumulated are chiefly confined to arthropods and mollusks, in which copper is invariably present. As discovered by Mendel and Bradley (1905), zinc is likewise present in relatively large amounts in at least one form of mollusks (*Sycotypus*), though the examination of various other marine animals—*Mytilus edulis*, *Modiola plicatula*, *Argina pexata*, *Eupagurus pollicaris*, *Ostrea virginiana*, and *Cancer irroratus*—failed to reveal appreciable amounts of this metal. According to these investigators, traces of zinc seemed to be present usually in *Urosalpinx cinerea*. In the liver of *Sycotypus* copper comprises about 8 per cent of the total ash, or 1.2 per cent of the dry tissue; while zinc comprises about 15 per cent of the ash, or 1.7 per cent of the dry tissue.

Because of the incompleteness of the data on the occurrence of copper and zinc in many forms of marine life, and the ease with which these animals may be procured in this locality, we have undertaken a quantitative study of their copper and zinc content. We have confined our work chiefly to fishes, with a few comparative data upon cœlenterates, mollusks, and arthropods. In the present paper we present the copper findings only, and will report the data upon zinc in a subsequent communication.

Methods.

In the present work copper was estimated in specimens prepared by thoroughly grinding the entire animals soon after they were removed from the water. The fishes were hashed without preliminary evisceration or scaling, and the crabs and shrimps without removal of the protective coverings. The oysters and clams were shucked. We expect to attempt detailed examinations of the tissues and organs in certain species later, but preliminary to subsequent investigations we desired comparative information concerning the copper content of the entire individuals.

In the estimation of copper we have tried several procedures, and finally adopted a colorimetric method based upon the familiar "blue test" with an excess of ammonia. A similar procedure was recommended by Heath (1897) in the analysis of tailings and lean blast furnace slags, but we have found it necessary to modify the conditions suggested by him to meet our special needs. Despite the fact that the method is an exceedingly long and tedious one, we have used it in preference to a shorter gravimetric or electrolytic process because of the difficulty of accurately weighing such small quantities of copper as those with which we have had to deal. It has been our experience that great care must be taken in following every detail of the procedure as adopted by us, in order to avoid the introduction of errors. We therefore describe the method in full without claiming any originality for it.

Preparation of Standard Copper Solution.—About 2 gm. of electrolytic copper are washed in dilute nitric acid to remove the trace of oxide, and then successively in water, alcohol, and ether. The dry metal is accurately weighed, and dissolved in 10 to 15 cc. of concentrated nitric acid. 5 cc. of

concentrated sulfuric acid are added, and the solution is heated on the water bath until the nitric acid is entirely removed. The solution is then diluted with about 200 cc. of distilled water, ammonia cautiously added until the reaction is just faintly acid, and the clear blue preparation diluted until each cubic centimeter contains exactly 1 mg. of copper.

Oxidation of the Tissue.—The weighed material (usually 100 gm.) is introduced into a porcelain dish or casserole, treated with 20 cc. of concentrated sulfuric acid, and slowly evaporated on a sand bath to a crisp charred mass, care being taken to prevent loss through spattering. The dry residue is allowed to cool, and treated with concentrated nitric acid, using the latter to wash down the sides of the vessel. 5 to 10 cc. of nitric acid are usually sufficient for the purpose. The vessel is again subjected to careful heating until the nitric acid is driven off. If necessary, this process is repeated two or three times, or until the quantity of charred material is considerably reduced, and may be transferred to a large (80 to 90 cc.) crucible.

This transfer is made as follows: the major portion of the material is carefully scraped into the crucible with a glass rod, the dish washed with the minimum quantity of dilute nitric acid, and the last traces of charred residue are carefully wiped from the sides of the dish with a small piece of ash-free filter paper. The washings and paper are added to the contents of the crucible, and the whole is evaporated to dryness on a water bath. The crucible is baked for a few minutes on a sand bath to remove the last traces of nitric acid, and ignited over a Bunsen burner at a dull red heat until a white or gray ash (blue if much copper is present) is obtained. If the charred material is large in amount, it is convenient to ash directly that which is scraped from the dish, and to add the washings and filter paper afterwards with subsequent evaporation and ignition. Occasionally, concentrated nitric acid must be added two or three times toward the last of the operation in order to secure complete oxidation of the organic matter. In this manner no difficulty has been experienced in obtaining an ash free from organic material.

Precipitation and Estimation of Copper.—The ash prepared as described above is extracted repeatedly with small quantities of hot dilute hydrochloric acid, the extracts are poured through a good ashless filter paper, and the residue and paper are thoroughly washed with boiling water. If the combined filtrate and washings exceed 50 cc., the solution is evaporated to approximately this volume. Otherwise it is neutralized directly with ammonium hydroxide, and again acidified by the addition of 1 or 2 cc. of concentrated hydrochloric acid.[1] A strong current of hydrogen

[1] In the analysis of certain marine animals, notably those having a chitinous protective covering, the ash contains a large quantity of calcium sulfate which is partly dissolved by the repeated acid extractions. Under these conditions a precipitation of calcium sulfate not infrequently occurs when the solution is concentrated, or neutralized and reacidified with hydrochloric acid. This necessitates an additional filtration before the sulfide precipitation.

sulfide is passed into the hot solution for 10 to 15 minutes, and the flask heated on the water bath until the sulfide precipitate settles. The latter is rapidly filtered off and washed with dilute hydrochloric acid (approximately 0.2 N in strength) which has previously been saturated with hydrogen sulfide.

The filter paper containing the sulfide precipitate is returned to the original flask in which the precipitation occurred, and repeatedly boiled with small portions of 2 N nitric acid to dissolve the copper. Each extract is decanted through a filter paper, the residue finally transferred to the paper, and washed with boiling water until the washings are neutral in reaction. The combined filtrate and washings are evaporated to dryness in a small casserole, and the residue is baked for a few minutes at a dull red heat to oxidize the trace of organic matter (or sulfur) which at this point is almost invariably present. The casserole is allowed to cool, and the cupric oxide dissolved in a few drops of concentrated nitric acid. A perfectly clear blue solution should result. If unoxidized matter is still present, as indicated by a slight yellow or brown color in the solution, the evaporation and baking must be repeated. Occasionally this process must be repeated three times.

When the copper nitrate is free from interfering matter, it is treated with 1 to 2 cc. of concentrated sulfuric acid, and carefully heated until white fumes are given off. The copper sulfate so obtained lends itself readily to the colorimetric estimation. For this purpose the contents of the casserole are diluted with a few cubic centimeters of distilled water, and washed into a 50 cc. Nessler jar. 20 cc. of strong ammonium hydroxide are cautiously added,[2] the solution is diluted to the 50 cc. mark, and mixed. The copper content is determined by running the standard copper sulfate solution into another Nessler jar containing 2 cc. of concentrated sulfuric acid, 20 cc. of distilled water, and 20 cc. of strong ammonium hydroxide, until the two solutions manifest exactly the same depth of color when examined longitudinally against a white background. The burette reading indicates the milligrams of copper in the sample taken for analysis.

If the copper content of the sample is greater than 8 mg., it is advisable to dilute the contents of the Nessler jar to a suitable volume with ammonia (1 volume of strong ammonium hydroxide to 3 volumes of distilled water), and use an aliquot part for the titration. Two closely agreeing titrations should be made against each unknown.

Analyses of aqueous copper solutions carried through the entire process, and of ground meat to which known amounts of copper were added, indicate that the maximum error in the use of the method is approximately .5 per cent, as indicated in Table

[2] The large excess of ammonium hydroxide is necessary to produce a clear permanent blue color, rather than the purple or purplish blue which results with smaller amounts of the base.

II. This is about as small an error as could be expected in the estimation of such traces of copper, especially in view of the fact that the destruction of the tissue, which is necessary in any procedure, is the most probable source of loss.

TABLE II.

Recovery of Copper from Known Mixtures.

Experiment No.	Material.	Cu present.	Cu added.	Total Cu.	Cu found.
		mg.	*mg.*	*mg.*	*mg.*
1	100 cc. of distilled water.	0	5.0	5.0	5.0
2	100 " " " "	0	10.0	10.0	10.0
3	50 gm. of meat.*	0	5.0	5.0	4.85
4	50 " " "	0	10.0	10.0	10.2
5	50 " " "	0	20.0	20.0	20.8
6	50 " " "	0	2.0	2.0	1.95
7	50 " " "	0	16.0	16.0	15.6
8	100 " of oysters.†	4.52	3.0	7.52	7.1
9	100 " " " ‡	6.00	5.0	11.0	10.7

* Preliminary analysis showed that 50 gm. of the meat did not contain a detectable amount of copper.

† No. 2 of Table III.

‡ No. 4 of Table III.

EXPERIMENTAL.

Using the above method, we have determined the copper in about thirty-five specimens of marine animals, the results of which are tabulated in Table III. The figures recorded are in most cases the averages of closely agreeing duplicates. With the exception of two analyses, one upon a large variety of clam, and the other upon a pigfish, copper has been found present in every case. Where we were unable to detect copper, the amount of tissue available for analysis was rather small, and we have been unable to procure other specmens of these species for duplicate determinations.

Of the fishes, the common sea-catfish contained the least copper, only minute traces (less than 1 mg. per kilo) being found in any of the three specimens analyzed. One sample of croaker (No. 14) showed less than 1 mg. of copper per kilo of tissue, while a second specimen (No. 26) showed 2.5 mg. per kilo. In

TABLE III.

Quantity of Copper in Marine Animals.

Analysis No.	Animal.	Amount taken for analysis.	Cu found.	Cu per kilo.	Remarks.
		gm.	*mg.*	*mg.*	
	Cœlenterata.				
9	Jellyfish.....	200	0.30	1.5	Entire animal ground.
42	Portuguese man-of-war.....	272	0.60	2.2	One entire animal.
44		81	0.30	3.7	Two small individuals.
	Pelecypoda.				
2	Oysters.....	100	4.52	45.2	Shucked.
4	"	100	6.00	60.0	
31	"	100	4.30	43.0	
32	"	100	2.40	24.0	
37	"	100	4.70	47.0	
39	Clams.....	77	0.0	0.0	
	Crustacea.				
1	Shrimps.....	100	1.70	17.0	Includes the protective coverings.
49	"	100	1.40	14.0	
3	"	100	0.80	8.0	
5	Crabs.....	100	0.65	6.5	
10	"	100	0.50	5.0	
	Elasmobranchii.				
29	Torpedo ray.	100	0.35	3.5	Three ground and sampled.
16	Sting ray....	100	0.52	5.2	Entire animal ground.
	Telestomi.				
6	Mullet.....	100	0.28	2.8	One or more individuals thoroughly ground and mixed before sampling.
15	"	100	0.20	2.0	
19	Angelfish.....	59	0.25	4.2	
11	Sea-catfish...	100	Trace.	Trace.	
20	" ...	100	"	"	
24	" ...	100	"	"	
14	Croaker.....	100	"	"	
26	"	100	0.25	2.5	
23	Pigfish.....	43	0.0	0.0	
25	Whiting.....	100	0.10	1.0	
28	Yellow-tail...	94	0.25	2.7	
12	Flounder.....	100	0.22	2.2	
27	"	100	0.25	2.5	
18	Spotted trout.	100	0.18	1.8	
17	Redfish.....	100	0.20	2.0	
21	Drum.....	86	0.25	2.9	
22	"	100	0.25	2.5	
30	Tarpon.....	100	0.24	2.4	

general the fishes contain a low but rather constant amount of copper, averaging about 2.5 mg. per kilo of tissue. The two species of cœlenterates—the common jellyfish and the Portuguese man-of-war—contain about the same proportion of copper in the fresh tissue as do the fishes, though the tissues of the former have a much higher water content. On a dry weight basis the cœlenterates would manifest a much higher copper content than the fishes.

Oysters were by far the richest in copper of the animals examined, the content varying from 24 to 60 mg. per kilo. These figures are, however, considerably lower than those obtained by Nelson (1915), who found that blue oysters contained 40 mg. of copper each and that uncolored ones contained 9 mg. each. In their extended study of the copper content of these mollusks, Hiltner and Wichmann (1919) report figures ranging from 6 to several hundred mg. per kilo, and state that oysters grown in water highly contaminated with metallic wastes from smelters and other factories contain the larger proportions of this metal. These authors are inclined to believe that the copper exists in the blood of the oyster, as in other mollusks, since the blood-containing fluid drained from shucked oysters invariably shows the presence of copper. Herdman and Boyce (1898–99) have demonstrated by microchemical tests that in certain oysters copper may occur in the leucocytes.

We have made one quantitative determination of the distribution of copper in fresh oysters. For this purpose 300 gm. were separated by careful dissection into four fractions composed of (*a*) mantles, (*b*) digestive glands or hepatopancreases, (*c*) adductor muscles, and (*d*) the other parts including the gills, which we have called the "residue." These fractions were weighed and analyzed separately, the results of which are shown in Table IV. The data indicate that the copper is rather uniformly distributed. No marked differences in the four fractions were found except in the muscle, which contained only about half as much copper as the other tissues. The residue was somewhat higher than the fractions composed of digestive organs and secreting mantles, possibly due to the vascularity of the gills which were contained in this fraction.

To determine whether copper exists in oysters in a diffusible form, the following experiment was made. A sample (87.5 gm.) of oysters, No. 37, containing 4.1 mg. of copper, was thoroughly ground, preserved with toluene, and dialyzed against distilled water. After 26 hours the dialysate was removed, and the experiment continued with fresh distilled water for 70 hours. Approximately 3 liters of water were used in all. Analysis of the combined dialysates showed that 0.5 mg., or 12.2 per cent of the total copper, had diffused. Hiltner and Wichmann (1919) believe that oysters store copper in excess of their physiological needs. A

TABLE IV.

Distribution of Copper in Oysters.

Tissue.	Amount taken for analysis.	Cu found.	Cu per kilo.
	gm.	*mg.*	*mg.*
Mantles	84.0	3.00	35.7
Digestive glands	56.7	1.95	34.4
Adductor muscles	38.5	0.70	18.2
Residue	102.0	4.10	40.2
Total	281.2*	9.75	34.7

* 300 gm. of fresh oysters were used for this experiment, but small fragments of tissue were unavoidably lost during the dissections, and considerable evaporation of water occurred. These factors account for the decrease of 18.8 gm. in weight.

similar opinion is held by several investigators concerning the storage of copper in the livers of gastropods. Perhaps the diffusible metal in our specimen represented the stored surplus; or it may be that the copper exists ordinarily in a very loose combination with protein, such as was found to be the case in the hemocyanin of *Limulus* by Alsberg and Clark (1910–11).

As shown in Table III, the shrimps and crabs had copper contents intermediate between oysters and fishes, the shrimps averaging about twice as much copper as the crabs, though differing considerably in the three samples examined. In a distributional test upon shrimps, the protective coverings were removed from 200 gm. and the hulls (including the heads and tails), and the meats were analyzed separately. The hulls amounted to 89.0 gm. and the meats to 104.5 gm., 6.5 gm. of water having been lost

through evaporation during the hulling. Contrary to our expectation, the hulls showed 7.3 mg. of copper per kilo, while the meats contained only 2.4 mg. per kilo, indicating that most of the copper in the shrimp is located in the protective covering.

DISCUSSION.

We believe that the data presented above clearly indicate that copper is a normal—possibly an essential—constituent, not only of mollusks and arthropods, where it is known to serve as an important ingredient of the respiratory protein, but of the tissues of fishes as well. Whether it exists in the latter as hemocyanins, or as other compounds of physiological importance, cannot at present be stated. It is interesting to observe that in the organisms examined by us the amounts of copper progressively diminish as we ascend from the comparatively simple to the more complex forms of life, and that when we come to the fishes, which are provided with hemoglobin, copper persists at a low but rather constant level.

In view of the almost universal occurrence of copper in marine animals it is not difficult to understand its source in fishes. Living largely at the expense of crustaceans, mollusks, and other low forms, fishes have an abundant supply of the metal in their diets. We believe that the universality of its occurrence and the constancy of its amount speak against its being an accidental constituent, or one due to the trace of copper in sea water. Hiltner and Wichmann (1919) report two analyses of sea water made by the Bureau of Chemistry laboratory at New York. In one, no copper could be detected in 3,500 cc.; in the other, a trace was detected in 4,000 cc. We have analyzed a specimen of water obtained about 30 feet from shore along the bathing beach of Galveston, where the maximum contamination would prevail, and found 0.14 mg. of copper per kilo of water. This figure is about one-twentieth of the average copper content of fishes. In a later communication we hope to present data on the distribution of this metal in some of the larger species.

SUMMARY.

1. Copper has been found to occur in two forms of cœlenterates; namely, the common jellyfish (*Aurelia*), and the Portuguese man-of-war (*Physalia*).

2. In oysters obtained locally, copper is rather uniformly distributed throughout the tissues as indicated by separate analyses of the mantle, digestive gland (hepatopancreas), large adductor muscle, and the remaining parts (residue) including the gills. The chief difference in the proportion of copper occurs in the muscle, where only half as much of the metal is found as elsewhere in the organism. Part of the copper in oysters (12.2 per cent in our experiment) exists in a diffusible form.

3. Analyses of twenty specimens of common fishes showed the presence of copper in every case except one, and the failure to detect it in this case was probably due to the small amount of material available for analysis. The average copper content was approximately 2.5 mg. per kilo.

4. It is believed that copper is a normal—possibly an essential—constituent of marine animals, and not an accidental one due to the trace of this metal in sea water.

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A STUDY OF THE FACTORS WHICH INTERFERE WITH THE USE OF YEAST AS A TEST ORGANISM FOR THE ANTINEURITIC SUBSTANCE.

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In a recent paper Williams[1] described a method of testing for the presence of the antineuritic substance which in this laboratory has been generally referred to as water-soluble B. For this purpose yeast was employed, and the basis of the test was the assumption, verified by experimental trials by Williams, that the yeast cell is capable of multiplication only when the antineuritic substance is present in its nutrient medium. By means of a series of dilutions of solutions which he used for the cultivation of yeast he was able to obtain results which gave promise of his method being useful in a quantitative sense as well as qualitative. Owing to the fact that the test as described by Williams could be carried out with very small amounts of material, and in a period of time much shorter than is required for the demonstration of the presence of the antineuritic substance in a nutrition experiment on a mammal, the method proposed for utilizing yeast as an experimental organism seemed to offer great possibilities of usefulness to those who are engaged in the study of methods for the isolation and identification of this most interesting dietary factor.

The work reported in this paper had its origin in an effort to develop proficiency in the conduct of experiments with yeast by the Williams method.

Considerable inconvenience was experienced in carrying out the test, owing to the number of small particles which became detached from the pen, and which interfered with the counting of the number of cells. A modification of Williams' method was

[1] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

therefore introduced, in which a platinum syringe needle, having an opening at right angles to its axis, was used for depositing the droplets on the cover-slip. The needle was attached to a piece of glass tubing by means of a short piece of rubber tubing, and it was found that with this instrument the suspension of yeast cells could be easily and quickly distributed, without leaving any detritus in the droplets. It was found that a fairly uniform distribution of cells could be obtained by this method. The slides were incubated in the manner described by Williams.

Our results were characterized by lack of uniformity in the development of the yeast, and for this reason an attempt was made to substitute for the cover-slips used by Williams the ordinary blood counting chamber of the Levy type. In order to break up the clumps of yeast cells without injuring their vitality air was blown through the suspension instead of shaking the latter. The solution was then distributed in test-tubes, 5 or 10 cc. in each. The results by this technique were much more uniform. Table II shows on one hand how the cells frequently were distributed in the droplet method, and on the other how the same suspension behaved in the blood counting chamber method.

Williams called attention in his paper to the fact that many times it is impossible to count the number of cells after a period of growth on account of the fact that the cells grow in three dimensions. He found it necessary to count a part and estimate the probable number. By the counting chamber method it is not necessary to guess at the number of cells. If the suspension is too rich it may be diluted to such a point as facilitates the counting. Immediately before counting, a little formalin was added to the solution in order to kill the cells and avoid further growth. Using this technique we have attempted to test several substances known to be rich or poor in the dietary factor water-soluble B. We will first consider the results obtained by the extraction of wheat germ with hot water.

1 gm. of wheat germ was boiled with 50 cc. of water for 10 minutes (Extract 1), the residue was boiled again with 50 cc. of water for 10 minutes (Extract 2), and the second residue was boiled with 50 cc. of water for 10 minutes (Extract 3). The separation of the residue from the soluble part offered some difficulty due to the viscosity of the solution. In some cases we

have separated the solution from the undissolved part by means of a centrifuge and in others by filtration in an alundum crucible. Only by passing the solution through a Berkefeld filter could an approximately clear solution be obtained. Eight successive extractions were made.

These extracts were evaporated to the volume of 25 cc. so each 1 cc. corresponded to 0.04 gm. of wheat germ. 5 cc. of the control solution were put into test-tubes previously sterilized, and the first extract was added as shown in Table I.

TABLE I.

Tube No.	Control solution.	First extract.	Control solution.
	cc.	cc.	
1	5.0	0	Seeded with Fleischmann's yeast.
2	5.0	0.1	
3	5.0	0.2	
4	5.0	0.3	
5	5.0	1.0	

From these tubes we made a series of slides by the droplet method and ran a comparative series by the counting chamber method, the results of which are presented in Table II.

If we take into account only those droplets which contain one single cell, we observe that they give rise to very different numbers of cells by the end of an 18 hour incubation period. In those squares where the yeast cell was dead, or had reached the stage where budding had ceased, there was, of course, no multiplication.

Seeding 1.

1 cell gave rise to 3 cells.
1 " " " " 14 "
1 " " " " 27 "
1 " " " " 34 "
1 " " " " 62 "

Seeding 2 (Control Solution Plus 0.1 cc. of Wheat Germ Extract).

1 cell gave rise to 3 cells.
In the rest the single cells were dead or incapable of budding.

TABLE II.

*Yeast Cells Counted in Different Droplets.**

Tube 1.					Tube 2.					Tube 3.					Tube 4.					Tube 5.				
Before incubation.																								
1	2	**1**	0	0	0	**1**	2	2	**1**	**1**	2	**1**	**1**(?)	0	2	**1**	**1**	0	0	1	D†	**1**	0	0
D	3	0(?)	0	0	3	2	D(?)	3	2	2	**1**	3	2	0	3	**1**	2	2	D	0	2		3	0
1	2	2	D(?)	**1**	**1**	**1**	**1**	0(?)	2	**1**	2	2	0	0	2	**1**	**1**	2	**1**	0	**1**	2	**1**	3
D	**1**	**1**	**1**	**1**(?)	**1**	2	2	0	**1**	2	3	0	**1**	2	3	0	**1**	0(?)	**1**	0	0	**1**	2	**1**
0	**1**	**1**	**1**(?)	**1**	0	3	2	**1**(?)		**1**	**1**	2	**1**	2	4	0	**1**	0	0	**1**	2(?)	**1**	2	0
After incubation for 18 hrs.																								
1	500	**0**	0	0	0	**1**	0	130	0	**207**	252	**301**	**0**	0	2	**1**	**245**	0	0	**500**	500	**1**	0	
27	3	2	0	0	0	2	143	6	98	258	**1**	0	0	0	0	**1**	2	500	0	0	5			
1	2	0	0	0	**1**	**1**	0	254	143	**101**	2,000	0	0	0	2	**1**	**0**	1,000	**1,000**	0	**0**	342	**1,000**	500
19	**1**	**3**	**62**	**0**	**3**	2	31	0	**0**	2	290	0	**151**	1,500	440	0	**130**	500	**349**	0	0	**1,000**	0	**290**
0	**3**	**27**	**34**	**14**	0	0	41	**0**		**0**	**124**	394	**0**	300	1,000	0	**400**			**500**		**75**		0

* In those cases where we were successful in seeding with a single cell the entry is made in bold-faced type. In the table showing the growth of the cells, the bold-faced type represents the progeny of single cells.

† D = Dead.

Seeding 3.

1 cell gave rise to 101 to 301 cells.
2 cells " " " 252 " 2,000 "

Seeding 4.

1 cell gave rise to 130 to 1,000 cells.

Seeding 5.

1 cell gave rise to 75 to 1,000 cells.

On the other hand, when using the test-tubes which contain 5 to 10 cc. of control solution, to which different amounts of wheat germ extract were added as stated above, by using the

TABLE III.

Tube No.	First count.	Second count (after 18 hrs.).	Average.
	cells	*cells*	*cells*
1	21	23	22
2	132	92	112
3	214	198	206
4	342	308	325
5	602	684	643

counting chamber we have a greater chance of securing the same number of cells distributed evenly, as respects their vital conditions, and thus effect a diminution of the magnitude of error due to individual cell differences. This procedure, in fact, gave more comparable results.

By the counting chamber method, and using the same suspension already used with the droplet method, the results given in Table III were secured.

The largest variation in the series of countings on the same suspension was in Tube 5 (602 to 684—difference 82, about 13 per cent variation between the first and second counting).

It seems, therefore, that a more even and reliable result can be obtained by the use of this procedure than with the droplet method devised by Williams, even though the droplets were made by the platinum needle instead of a pen.

We have tested the effect of increasing the quantity of the first aqueous extraction of wheat germ on the growth of yeast. The uniformly beneficial effects were suggestive that the high content of water-soluble B in the extract exerted a stimulating effect on the growth of the yeast.

The results of testing the second and third extracts prepared from the residue of the first extraction when tested for their effect on the growth of yeast are given in Tables IV and V.

TABLE IV.

Test with Second Extract.

	No. of cells.		Average after 12 hrs. incubation.
Control solution	21		21
" " + 0.1 cc	132	92	112
" " + 0.2 "	214	198	206
" " + 0.4 "	342	308	325
" " + 1.0 "	602	684	643

TABLE V.

Test with Third Extract.

	No. of cells.		Average after 24 hrs. incubation.
Control solution	150	189	174.5
" " + 1.0 cc	378	366	372
" " + 2.0 "	421	480	450.5
" " + 3.0 "	511	554	532.5
" " + 1.0 " first extract	1,068	1,240	1,154
" " + 1.0 " second "	649	633	641

The results show clearly that there is a stimulating effect of the extracts on the rate of multiplication of yeast and that in general the greater the amount of extract added the more pronounced the acceleration.

Alcoholic extracts of wheat germ were also prepared in which various strengths of alcohol were used, varying from 40 to 75 per cent. In all cases the germ was boiled for 10 minutes in alcohol and the solution filtered through paper or an alundum filter, then evaporated on a water bath at low temperature with a fan blowing on the surface. In all cases it was observed that

the growth of yeast was markedly stimulated by the addition of an alcoholic extract of wheat germ to the control solution.

In some experiments we have used wheat germ extract treated with sodium bicarbonate and autoclaved, in order to destroy as much as possible of the antineuritic substance. Before addition of such extracts to the nutrient medium the sodium bicarbonate was neutralized with hydrochloric acid.

Extract of fresh beef made by boiling the latter with water was also employed. The beef muscle was treated with 2 per cent of $NaHCO_3$, moistened and allowed to stand for 3 hours, then autoclaved 1 hour at 15 pounds pressure. Extract of rolled oats was also used. The oats were treated with 2 per cent of $NaHCO_3$ and autoclaved at 15 pounds pressure as described for the meat muscle. The oats so treated were fed to young rats in a diet consisting of rolled oats 40.0, gelatin 5.0, casein 5.0, NaCl 1.0, butter fat 3.0, $CaCO_3$ 1.5 per cent. The animals failed to grow. After 41 days 3 per cent of untreated wheat germ was added to the diet to furnish the antineuritic substance. The animals responded at once with rapid growth. 40 per cent of untreated oats furnishes an abundance of antineuritic substance, water-soluble B, for growth. The treatment of the oats with alkali had, therefore, destroyed its content of this substance.

The series has been extended by the employment of meat extract prepared by precipitation with barium, the addition of barium being stopped while the solution was still distinctly acid. After the removal of the barium sulfate the remaining acidity was neutralized by ammonium hydroxide.

All extracts employed in our experiments were carefully sterilized by boiling and kept in an ice box until used. Observations were made to establish that there was no contamination of these extracts with bacteria.

Table VI illustrates the effect of 40 per cent alcoholic extract of wheat germ added to the control solution on the rate of development of yeast cells. Equal amounts of successive extracts were employed.

Table VII illustrates the effect of adding 1 cc. of each of a series of eight successive extracts of wheat germ, using 40 per cent alcohol as a solvent.

Table VIII illustrates clearly the progressively increasing benefit to the yeast by adding increasing amounts of 75 per cent alcoholic extract of wheat germ.

TABLE VI.

	No. of cells after 18 hrs.		Average.
5 cc. of control solution	147	133	140
5 " " " " + 1 cc. first extract.	811	798	804.5
5 " " " " + 1 " second "	507	509	508
5 " " " " + 1 " third "	392	416	404
5 " " " " + 1 " fourth "	430	399	414.5

TABLE VII.

	No. of cells after 20 hrs.			Average.
5 cc. control solution	570	433	480	494.3
5 " " " + 1 cc. first extract	1,560	1,720		1,640
5 " " " + 1 " second "	1,610	1,720		1,665
5 " " " + 1 " third "	890	810		850
5 " " " + 1 " fourth "	760	670		715
5 " " " + 1 " fifth "	590	690		640
5 " " " + 1 " sixth "	820	680	780	760
5 " " " + 1 " seventh "	760	560		660
5 " " " + 1 " eighth "	500	600		550

TABLE VIII.

	No. of cells after 20 hrs. incubation per 400 squares.		Average.
5 cc. control solution + 1.0 cc. water	499	483	491
5 " " " + 0.9 " " + 0.1 cc. first extract	1,000	870	935
5 cc. control solution + 0.8 cc. water + 0.2 cc. first extract	1,360	1,390	1,375
5 cc. control solution + 0.6 cc. water + 0.4 cc. first extract	1,610	1,740	1,675
5 cc. control solution + 1.0 cc. water + 1.0 cc. first extract	2,180	2,540	2,360

Tables IX, X, and XI illustrate the effect of adding to the control solution aqueous or alcoholic (40 per cent alcohol) extracts of wheat germ. In these experiments the wheat germ had

TABLE IX.

	No. of cells after 18 hrs. per 400 squares.		Average.
5 cc. control solution	770	580	675
5 " " " + 1 cc. autoclaved wheat germ extract	2,460	1,860	2,160
5 cc. control solution + 2 cc. autoclaved wheat germ extract	2,650	3,530	3,090
5 cc. control solution + 1 cc. alcoholic extract of wheat germ	2,520	2,420	2,470
5 cc. control solution + 2 cc. alcoholic extract of wheat germ	2,530	2,320	2,425

TABLE X.

	No. of cells after 18 hrs. per 400 squares.		Average.
5 cc. control solution	160	210	185
5 " " " + 1.5 cc. water + 0.5 cc. 75 per cent alcoholic extract	1,340	1,210	1,275
5 cc. control solution + 1.0 cc. water + 1.0 cc. 75 per cent alcoholic extract	2,060	2,030	2,045
5 cc. control solution + 1.0 cc. water + 0.5 cc. 75 per cent alcoholic extract + 1.0 cc. autoclaved wheat germ	1,920	1,480	1,700
5 cc. control solution + 1 cc. autoclaved wheat germ + 1 cc. 75 per cent alcoholic extract	2,790	3,920	3,355
5 cc. control solution + 1 cc. autoclaved wheat germ + 1 cc. water	2,190	2,120	2,155

TABLE XI.

	No. of cells after 20 hrs. per 400 squares.		Average.
5 cc. control solution + 2 cc. water	100	120	110
5 " " " + 1 " " + 1 cc. extract autoclaved wheat germ	2,440	2,320	2,380
5 cc. control solution + 2 cc. extract autoclaved wheat germ	2,730	2,630	2,680
5 cc. control solution + 1 cc. water + 1 cc. 40 per cent alcoholic extract wheat germ	2,040	2,320	2,180
5 cc. control solution + 2 cc. 40 per cent alcoholic extract wheat germ	2,190	1,670	1,930

been treated with 2 or 4 per cent sodium bicarbonate, allowed to stand in a moist condition for 3 hours or longer, in order that the alkali might penetrate the material. The wheat germ was then autoclaved at 15 pounds pressure for 3 hours, the acid neutralized, and the material dried in a current of warm air.[2]

TABLE XII.

	24 hrs.		48 hrs.	
	Counts.	Average.	Counts.	Average.
1. 10 cc. control solution.	300 310	305	2,350 2,500	2,425
2. 10 " " " + 1 cc. 10 per cent glucose.	390 330	360	5,020 4,940	4,980
3. 10 cc. control solution + 2 cc. 10 per cent glucose.	360 380	370	8,300 7,650	7,975
4. 10 cc. control solution + 1 cc. autoclaved wheat germ extract.	1,540 1,350	1,445	2,880 3,060	2,970
5. 10 cc. control solution + 1 cc. autoclaved wheat germ extract + 1 cc. 10 per cent glucose.	1,400 1,390	1,395	3,780 4,020	3,900
6. 10 cc. control solution + 1 cc. 75 per cent alcoholic wheat germ extract.	1,650 1,810	1,730	5,340 5,450	5,395
7. 10 cc. control solution + 1 cc. 75 per cent alcoholic wheat germ extract + 1 cc. 10 per cent glucose.	1,570 1,540	1,555	5,400 5,760	5,580

It is easily apparent that the addition of these extracts exerted a profound stimulating effect on the growth of the yeast, but that the extent of acceleration of the growth of yeast is not proportional to the amount of extract added.

[2] This germ was used in a feeding experiment with rats to show whether it still contained any appreciable amount of water-soluble B. The ration consisted of casein 18, treated wheat germ 15, butter fat 5, salt mixture (No. 185) 3.7, dextrin 58.3 per cent. The animals failed to grow. In a subsequent period rapid growth took place when 3 per cent of untreated germ was included in the diet. All the antineuritic substance had been practically destroyed by heating with alkali.

Table XII illustrates the effect on the growth of yeast of adding to the control solution varying amounts of glucose and of glucose together with extracts of wheat germ.

It will be observed that Nos. 2 and 3 show a surprising acceleration due to the addition of double the amount of glucose. 1 cc. of 10 per cent glucose solution added to 10 cc. of control solution produced 4,980 yeast cells after 48 hours. 2 cc. of the glucose solution added to 10 cc. of control solution led to the development of 7,975 cells in the same interval. Comparing Nos. 4 and 5, the addition of 1 cc. of autoclaved wheat germ extract produced 2,970 cells in 48 hours, whereas the same medium with 1 cc. of 10 per cent solution produced 3,900 cells. That results are not always consistent is shown by comparison of Nos. 6 and 7, in which there was a marked effect from the addition of 1 cc. of 75 per cent alcoholic extract of wheat germ, and very slight further stimulation by the addition of 1 cc. of 10 per cent glucose.

Table XIII illustrates further the lack of uniformity in the development of yeast under different conditions.

Comparing Nos. 2 and 3, greater growth was secured by adding to 10 cc. of the control solution 1 cc. of 10 per cent glucose solution than was obtained by the addition of 0.1 cc. of 75 per cent alcoholic extract of wheat germ without additional glucose. In order to test the possibility of securing comparable development of yeast when the cultural conditions for every cell were as nearly as possible identical the following procedure was tried.

A drop of yeast suspension was placed on a clean cover-slip, and the number of yeast cells contained in it carefully determined by counting. The yeast cells were then immediately washed into a sterile test-tube by means of control solution, and the amount of control solution was then adjusted so that 0.1 cc. of nutrient solution was available for each yeast cell present. The following five experiments illustrate the results of this procedure. An examination of Table XIV shows that in the first test-tube there were 21 yeast cells, each with 0.1 cc. of nutrient medium. After 12 hours the number was increased to 39, an approximate multiplication of the original number by 2. In the second tube 29 cells were present, and in 12 hours this had multiplied the original number by 16. In the third tube 31 cells were present, and after 12 hours this had multiplied the original number by 19

In the fourth tube 34 cells were present, and after 12 hours this had multiplied 21½ times the original number. In the fifth tube 43 cells were present and in 12 hours the original number was multiplied by 10. The cause of these irregular results is unquestionably to be attributed to the introduction of injured cells or cells of low reproductive power into the test specimens.

TABLE XIII.

	18 hrs.		52 hrs.	
	Counts.	Average.	Counts.	Average.
1. 10 cc. control solution.	350 290	320	4,680 4,100	4,390
2. 10 " " " + 1.0 cc. 10 per cent glucose.	420 380	400	4,660 4,620	4,640
3. 10 cc. control solution + 0.1 cc. 75 per cent alcoholic extract wheat germ.	620 670	645	3,600 3,760	3,680
4. 10 cc. control solution + 0.1 cc. 75 per cent alcoholic extract wheat germ + 1.0 cc. 10 per cent glucose.	910 980	945	6,260 5,320	5,790
5. 10 cc. control solution + 0.5 cc. 75 per cent alcoholic extract wheat germ.	940 1,300	1,120	4,240 4,400	4,320
6. 10 cc. control solution + 0.5 cc. 75 per cent alcoholic extract wheat germ + 1 cc. 10 per cent glucose.	930 890	910	4,340 4,452	4,396
7. 10 cc. control solution + 1 cc. 75 per cent alcoholic extract wheat germ.	1,250 1,800	1,525	4,420 4,590	4,505
8. 10 cc. control solution + 1 cc. 75 per cent alcoholic extract wheat germ + 1 cc. 10 per cent glucose.	1,130 1,240	1,185	4,380 4,190	4,285

We have sought to test the value of a procedure involving the addition of 1 drop of opalescent suspension of yeast cells in water to several test-tubes, each containing 10 cc. of nutrient solution, and then counting these cells in the Levy chamber before incubation.

Table XV illustrates one set of experiments in which fairly close agreement was obtained by this procedure, but the agreement is presumably due to the good fortune of selecting a series of cells all or nearly all of which were capable of development.

In Table XVI are recorded the results of a series of trials designed to determine the effect of various extracts on the rate of

TABLE XIV.

Tube No.	Original count.	Control solution.	Count after 12 hrs.	Average.
	cells	*cc.*	*cells*	*cells*
1	21	2.1	43 35	39
2	29	2.9	464 461	462.5
3	31	3.1	594 586	590
4	34	3.4	767 698	732.5
5	43	4.3	422 454	438

TABLE XV.

Tube No.	Count per 400 squares.	Count after 22 hrs.		Average.
	cells	*cells*		*cells*
1	3	186	198	192
2	4	174	195	184.5
3	3.5	169	153	161

development of yeast. The volume of solution was kept constant by the addition of glucose solution or water. The materials from which the extracts were prepared in these experiments had been subjected to heat under pressure in the presence of sodium bicarbonate as described in the methods of preparation of materials. In all cases these preparations had been tested with rats and found to be at least practically free from the dietary factor water-soluble B. Notwithstanding the fact that they con-

tained so little of this substance as to be undemonstrable by experiments on the rat the extracts were still very effective in stimulating the growth of yeast. These results suggest that although the addition of the antineuritic substance in minute amounts may have influenced the growth of yeast in these experi-

TABLE XVI.

	No. of cells after 18 hrs.		Average.
1. 9 cc. control solution. 1 " 10 per cent glucose solution. 1 " H_2O.	390	350	370
2. 8 " control solution. 2 " glucose " 1 " H_2O.	310	380	345
3. 8 " control solution. 2 " glucose " 1 " eighth extract wheat germ.	320	340	330
8 " control solution. 2 " dextrose " 1 " autoclaved wheat extract (first).	1,750	1,610	1,680
5. 8 " control solution. 2 " glucose " 0.5 " first extract wheat germ. 0.5 " H_2O.	1,880	1,750	1,815
6. 8 " control solution. 2 " glucose " 1 " first extract wheat germ.	1,960	1,990	1,975
7. 8 " control solution. 2 " glucose " 1 " rolled oats extract (made from rolled oats autoclaved with 2 per cent $NaHCO_3$).	1,980	2,010	1,995
8. 8 cc. control solution. 2 " glucose " 1 " beef extract (from beef steak autoclaved with 2 per cent $NaHCO_3$).	1,700	1,760	1,730

TABLE XVII.

	No. of cells after 20 hrs.		No. of cells after 43 hrs.		Gas.
	Counts.	Average.	Counts.	Average.	
					cc.
1. 9 cc. control solution. 1 " 10 per cent glucose solution. 1 " H_2O.	156 137	146.5	2,510 2,880	2,695	2.5
2. 8 " control solution. 2 " glucose " 1 " H_2O.	143 139	141	2,300 2,180	2,240	2.5
3. 8 " control solution. 2 " glucose " 1 " eighth extract wheat germ.	133 128	130.5	1,740 2,010	1,875	0.5
4. 8 " control solution. 2 " glucose " 1 " autoclaved wheat extract (first).	732	732	4,180 4,230	4,205	10
5. 8 " control solution. 2 " glucose " 0.1 " first extract wheat germ.	493 535	514	3,900 4,780	4,340	4
6. 8 " control solution. 2 " glucose " 0.5 " first extract wheat germ (alcohol). 0.5 " H_2O.	675	675	5,020 5,210	5,115	5
7. 8 " control solution. 2 " glucose " 1 " first extract wheat germ (alcohol).	1,340	1,340	4,740 4,100	4,420	8
8. 8 " control solution. 2 " glucose " 1 " fifth extract wheat germ (alcohol).	170 143	156.5	4,830 5,140	4,985	8
9. 8 " control solution. 2 " glucose " 1 " extract rolled oats (fresh oats).	636 752	694	3,680 4,310	3,995	8
10. 8 " control solution. 2 " glucose " 1 " steak extract (alkalinized steak).	1,027	1,027	4,560 4,940	4,750	10
11. 8 " control solution. 2 " glucose " 1 " third extract wheat germ (alcohol).	880	880	2,230 2,340	2,285	3

ments, this factor is so small as to make it practically certain that other added substances, as glucose and amino-acids, also served to influence the results in a marked way.

Table XVII illustrates the results obtained in a series of experiments in which it was sought to estimate by the gas produced by the yeast culture the extent of growth of the yeast in several

TABLE XVIII.

	No. of cells after 20 hrs.	Average.
1. 1 cc. control solution.	1,020 1,230	1,125
2. 1 " " " 1 " autoclaved wheat extract.	1,980 2,080	2,030
3. 10 " control solution. 0.5 " digested meat (prepared from fresh steak not autoclaved with alkali).	1,820 1,970	1,895
4. 10 cc. control solution. 0.5 " digested meat extract (prepared from fresh steak not autoclaved with alkali).	2,048 2,200	2,124
5. 10 cc. control solution. 1 " digested meat extract (prepared from fresh steak not autoclaved with alkali).	2,412 1,940	2,176
6. 10 cc. control solution. 1 " first extract wheat germ.	2,020 2,380	2,200
7. 10 " control solution. 0.5 " first extract wheat germ.	1,680 1,880	1,780

types of modified nutrient medium. These results are so variable, when we compare the gas production with the number of yeast cells generated during the period of incubation, that the measurement of the amount of gas produced does not give promise of value in this line of work.

In Table XVIII is presented a series of observations on the effect of adding an amino-acid mixture obtained by the hydrolysis of beef muscle with sulfuric acid.

So far as our knowledge permits us to judge, these digested meat extracts could serve only as a source of amino-acids, since the treatment was sufficient to reduce the proteins practically to the amino-acid state, and, since muscle tissue is, even in the fresh condition, very poor in the antineuritic factor (water-soluble B), the same meat after hydrolysis with strong sulfuric acid would scarcely contain a trace of it. Yet these meat digests exerted a profound influence on the rate of development of yeast, approximately doubling the number of cells which were counted after 20 hours incubation in Experiments 6 and 7, Table XVIII. It is again apparent that doubling the amount of the antineuritic substance derived from wheat germ extract does not exert a proportional stimulating effect on the growth of yeast.

The series of observations which we have presented above seem to us to form a conclusive demonstration that the use of yeast as a test organism for determining the presence or absence of the antineuritic dietary factor is complicated by so many disturbing factors as to make it of little if any value. Whenever extracts of natural foods are to be tested it is inevitable that food substances of one kind or another which greatly stimulate the growth of yeast must be added simultaneously with the unidentified dietary essential for which the test was designed. Glucose and amino-acids effect such stimulation

We do not desire to draw the deduction from the data here presented that the antineuritic substance is not necessary for the development of yeast, or that it does not stimulate the growth of yeast, but the several modifications of the procedure originally described for the utilization of yeasts as a test for the antineuritic substance point so definitely to several difficulties in the way of making this a satisfactory test that it has seemed to us desirable to present the results for the benefit of those who may be interested in this line of work.

HEMATO-RESPIRATORY FUNCTIONS.

VI. THE ALTERATION OF THE CO_2 RATIO (H_2CO_3 : $NaHCO_3$) IN THE BLOOD DURING ELEVATION OF BODY TEMPERATURE.

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(Received for publication, August 7, 1920.)

Increase in temperature lowers the solubility coefficient of CO_2 in blood. It also increases the tension of water vapor in the lungs, and this tends to reduce the partial pressure of CO_2. Both conditions act, therefore, to reduce the dissolved CO_2, and presumably the H_2CO_3, in the arterial blood. On merely physicochemical grounds we should, therefore, expect that increase in body temperature, whether from fever or from extrinsic causes, *e.g.* hot packs or baths, would depress respiration to a degree sufficient to allow the alveolar CO_2 tension to rise and thus counteract the reduction in the solubility of CO_2.

This, however, is not the case. On the contrary, rise of body temperature is accompanied by an augmentation of respiration and a fall in the alveolar CO_2 tension in excess of the decrease produced by the higher partial pressure of water vapor in the lungs. These physiological relations are to be seen in the following experiments. All of those performed *in vivo* were upon myself.

Experiment 1.—Subject, normal, human, adult, male. Body temperature was increased by immersion up to the chin in water at 43°C. for 20 minutes. Oral temperatures taken. Alveolar air obtained by Henderson sampler (1).

	Oral temperature.	Alveolar CO_2.
	°C.	*mm.*
Normal resting	37	39.9
After 20 min. immersion	40	35.0
Difference	+3	−4.9

The increase of water vapor in the lungs from 46.65 mm. at 37°C. to 54.87 mm. at 40°C., unless compensated in some way, would result in a fall of 0.5 mm. in CO_2 alveolar tension, or 0.07 per cent. The experimental findings here indicate a decrease in the dry gas percentage from the normal of 5.6 per cent to 4.9 per cent at 40°C., a fall of 0.7 per cent or 4.9 mm. CO_2.

Overbreathing of the amount thus indicated is not sufficient to effect in the brief period of such an experiment any considerable lowering of the available blood alkali, as expressed in the dissociation curve, although it would of course induce an immediate slight decrease of the alkali in use, as expressed by a shifting of the arterial point along the curve to the left. That the blood alkali is not sensibly lowered is to be seen in the following experiment.

Experiment 2.—General procedure as in previous protocol. Blood samples were drawn from the median basilic vein before and at the end of the period of immersion and were equilibrated at 37°C. with CO_2 at 40 mm. tension.

20 minutes immersion at 43.2°C.

	Oral temperature.	Alveolar CO_2.	CO_2 combined by blood at 40 mm. tension, 37°C.
	°C.	*mm.*	*vol. per cent*
Normal resting	37.0	39.2	47
After 20 min. immersion	40.8	33.9	47
Difference	+3.8	−5.3	0

The fall in alveolar CO_2 tension due to water vapor in this experiment would amount to 0.6 mm. An actual fall of 5.3 mm. occurred. This represents a decrease in dry CO_2 from the normal of 5.5 per cent to 4.8 per cent.

As indicated by the CO_2-combining power at 40 mm. CO_2 tension, 37°C., there was no absolute decrease in available blood alkali.

Experiment 3.—Normal blood of the same person equilibrated at 35° and at 40°C. with 39.5 and 38.7 mm. CO_2, respectively. Analysis and calculation showed the CO_2 content, dissolved and combined CO_2, and CO_2 ratio as follows:

	CO_2 content.	CO_2 dissolved.	CO_2 combined.	CO_2 ratio.
	vol. per cent	*vol. per cent*	*vol. per cent*	*per cent*
At 35°C. and 39.5 mm. CO_2.....	48	2.90	45.10	6.4
" 40 " " 38.7 " CO_2.....	47	2.58	44.42	5.8

Increase in body temperature results in a considerable fall in the alveolar CO_2, and, therefore, probably in the CO_2 dissolved in the arterial blood. This decrease, as is shown in Experiment 2, is not compensated by a fall in the available alkali of the blood, for this remained constant. Unless some alteration in the CO_2-carrying power of the blood occurs in response to change in temperature, a displacement to some new value in the balance between the free and combined CO_2, the CO_2 ratio, of the arterial blood must occur and, presumably, a change in C_H result.

That such a change does occur in the CO_2-combining power of the blood but that it is of a magnitude insufficient to compensate the fall in dissolved CO_2, is shown in the following section.

The Influence of Temperature Upon the CO_2-Carrying Power of Blood in Vitro.

Christiansen, Douglas, and Haldane (2) cite one equilibration to illustrate the effect of temperature upon the CO_2-combining power of defibrinated blood. Van Slyke, Stillman, and Cullen (3) have shown the extent of this change in plasma. The quantitative relations for whole blood, however, have not been made clear. That temperature does influence to some extent the equilibrium between carbon dioxide and the buffer substances of the blood, independent of the effect on carbon dioxide solubility, has been shown by L. J. Henderson (4).

The greatest determining factor, however, in placing the level of alkali in use, must be the dissolved CO_2 in relation to the dissociation curve. In order to test quantitatively the degree of response of alkali to the dissolved CO_2, dissociation curves were plotted from normal dog blood at temperatures of 17°C. or 20° and 40°C. The curves so obtained were then compared at those points which represented like amounts of CO_2 in solution. Coincidence at these points would indicate a complete response

of the alkali to the dissolved CO_2 independent of other effects of temperature.

Normal blood from four dogs ((*a*), (*b*), (*c*), and (*d*)) was oxalated and then equilibrated at 40°C. with CO_2 at three tensions. A second portion of the same blood was then equilibrated at 17°C., or at 20°C., with CO_2 at three tensions.

The CO_2 retained by the blood after each equilibration minus the CO_2 in solution is given in Table I.

TABLE I.

Blood.	Temperature 17°C.			Temperature 40°C.		
	Equilibrating tension CO_2.	Combined CO_2.	Dissolved CO_2.	Equilibrating tension CO_2.	Combined CO_2.	Dissolved CO_2.
	mm.	*vol. per cent*	*vol. per cent*	*mm.*	*vol. per cent*	*vol. per cent*
(*a*)	22.4	39.35	2.65	21.2	28.61	1.39
	41.0	49.15	4.85	38.8	35.45	2.55
	74.6	63.18	8.82	70.5	49.37	4.63
(*b*)	22.4	29.35	2.65	21.2	18.61	1.39
	41.0	35.15	4.85	38.8	28.45	2.55
	74.6	45.12	8.82	70.5	37.37	4.63
	Temperature 20°C.			Temperature 40°C.		
(*c*)	16.1	36.12	1.80	15.2	24.00	1.00
	40.8	50.23	4.73	38.8	39.45	2.55
	81.6	60.45	9.55	77.6	49.88	5.12
(*d*)	16.1	30.12	1.80	15.2	22.00	1.00
	40.8	42.23	4.73	38.8	34.45	2.55
	81.6	50.45	9.55	77.6	43.88	5.12

The curves from Bloods (*a*) and (*b*) are plotted in Fig. 1. The close coincidence of the curves at 17° and 40°C. at those points which represent like amounts of CO_2 in solution is apparent. The curves at 40°C. tend to run slightly lower than those at 17°C., but hardly in excess of the error of the method.

Rise in body temperature from all the aspects approached in this paper tends to displace to a new value the ratio of H_2CO_3: $NaHCO_3$ in the blood.

In order adequately to compensate the fall in dissolved CO_2 the dissociation curve would need to be of such a shape that all values of alkali maintain a constant ratio with the dissolved CO_2. The curves are entirely too flat to allow this, and the C_H is therefore decreased.

These observations tend to confirm the supposition that under increase in body temperature the CO_2 ratio is reduced by physiological, even more than by mechanical, factors.

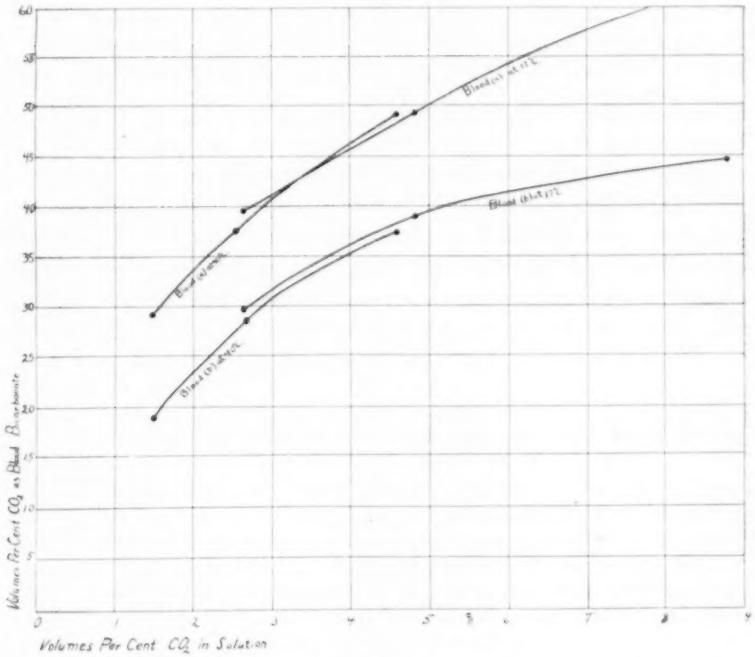

FIG. 1.

SUMMARY.

Rise in body temperature lowers the alveolar CO_2 tension and reduces the amount of CO_2 in solution in blood.

No compensatory changes in the CO_2-combining power of the blood occurs.

The alkali in use is not lowered in proportion to the decrease in dissolved CO_2.

The CO_2 ratio is therefore reduced and, unless there is some alteration in the dissociation constants of the blood, the C_H is presumably lowered (5).

In vitro temperature affects the dissociation curve of blood solely by altering the solubility of CO_2 and concentration of H_2CO_3. The concentration of H_2CO_3 which produces a certain amount of $NaHCO_3$ at 17° or 20°C. produces the same amount at 40°.

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THE NUTRITIVE PROPERTIES OF MILK, WITH SPECIAL REFERENCE TO REPRODUCTION IN THE ALBINO RAT.

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The nutritive efficiency of the various known and unknown constituents of milk has been tested out repeatedly on different types of experimental animals, particularly on the rat, usually by adding milk or one of its constituents to a basal ration deprived in whole or in part of the substance in question, less often by using milk as a basal ration and superimposing other substances upon it. It is unnecessary to review extensively the compendious literature on this subject since recent papers on particular phases contain abundant references.

The relative value of the milk proteins is of a high order (1–3) although under certain conditions lactalbumin, supposedly the most complete of all the milk proteins, has appeared not to be so (4, 5). Milk sugar, long known to possess desirable characteristics not found in the other disaccharides, has been shown to be the carrier of water-soluble vitamine unless carefully purified (6, 7) and a complementary relation of lactose to lactalbumin has been suggested (5). Milk fat occupies its unique position by virtue of possessing fat-soluble vitamine (8, 9), whose nature and functions in the organism are still an unsolved problem (10), and whose origin and distribution in nature continue to occupy the interest of a number of investigators. As great a mystery surrounds the nature and function of the water-soluble vitamine of milk, first brought to light in the work of Hopkins (11) and used in the form of "protein-free milk" by Osborne and Mendel in a long series of investigations. The question as to its identity with the water-soluble vitamine from other sources, notably yeast, was raised several years ago (12, 13) and has been renewed in a recent critical paper (14); evidence has also been presented to show that the water-soluble vitamine of milk may indeed be unique and different from that present in other foodstuffs (15). The antiscorbutic value of milk has been demonstrated (16) although this may sometimes be inconsiderable. The inorganic salts of milk are successfully used as a source of mineral requirement of the rat (17).

Incidental to and growing out of the above have been investigations as to the effect of heat, storage, and other factors on the various constituents, notably the proteins (13, 18), the fat-soluble (19–21), and antiscorbutic vitamines (16, 22). A quantitative measure of these effects is necessary if we are to know the absolute requirements of the experimental animal and the adequacy of milk to meet them; for neither of these quantities is there as yet an absolute measure, at least not as regards the vitamines. Since it is probable that the amount of vitamines present in milk is dependent among other things on the amounts present in the feed of the animal giving the milk (23) and this in turn upon seasonal variations (22) and stage of maturity of the plant (24), we are obviously still far from a definite answer to these questions.

As the first and sole nourishment of the young animal milk is considered a perfect food and certainly no other single product of animal or vegetable life used for food so fully provides for the nutritive needs of growing (and adult) animals as does milk. Indeed many of these requirements were not recognized until it was discovered that the addition of milk or of its derivatives could correct faulty diet. After a young animal is weaned its nutritive demands are met by a variety of food materials among which, except in the case of man and his domestic animals, milk is not found. The new foods to which an animal is introduced after weaning are generally characterized by a lower protein content, a wider variety of mineral sources, and by the addition of a new type of material, roughage. The interesting question presents itself as to whether milk could continue to be an adequate food for the whole span of life or whether it is lacking in some factor which is necessary for the normal performance of physiological function and for the fulfillment of the normal expectancy; whether it possesses in too great abundance a factor which would prevent normal behavior either because of a consequent improper balance among the ingredients or through bringing about early cessation of function of a gland or organ. In the answer to this question might also be found some indication of the variation in food requirements during the different periods or cycles into which the life of an animal may be divided; any deterioration which milk might suffer in the process of evaporation or desiccation, if not made obvious during brief periods of experimentation, might thus be brought to light, under prolonged scrutiny.

Practically all our information on milk concerns itself with cow's milk and its derivatives; excepting the experiments on scurvy, the animals whose behavior forms the basis of the conclusions drawn are mostly rats. In her comprehensive book on milk (25) Lane-Claypon devotes several chapters to the nutritive value of milk and the data then available from experiments on various species of animals with milks from the same and other species. All these investigations were of relatively short duration, in very few of them was milk the sole food, and the conception of vitamines, then in its earliest infancy, had not yet modified the entire aspect of nutritive values. The difficulties of such animal experimentation are also pointed out with particular emphasis on the dissimilarity of milks, the complete absence of information as to the composition of milk of some species, differences in the condition of young animals at birth, different rates of growth in different species, a varying rate of growth in the same species with the consequent changing demands of the organism, and the great factor of variability.

The present investigation of which this is a preliminary report was begun to secure information on the food value of cow's milk and its products from the usual type of laboratory animals, several of which have thus far been under observation. This paper includes only the first findings obtained from rats.

EXPERIMENTAL.

Young rats from our stock colony were placed on the experimental rations at the time of weaning, two in a cage and usually mated from different litters. Their average age at this time was 25 days, and only three of the series were as old as 35 days. Their average weight was about 30 gm., and a few from small litters weighed as much as 40 or 50 gm. The fresh milk used was Grade B raw and was placed in the cages each morning in amounts as indicated by the previous day's consumption. Next morning the amount remaining, if any, was measured back. Usually this was very little and it was found sweet to the smell as often as sour, showing that contamination, while probably always present, was not extensive. The dry milk food[1] was renewed

[1] Ambrosia and Klim were obtained from the Mammala Corporation and the Merrell-Soule Company, respectively.

every 2nd or 3rd day, and while scattering was common it was possible to secure a fair idea of food consumption especially when the amounts supplied closely approximated the requirement. Water was always available in inverted tubes. The animals were weighed twice a week until fairly grown, thereafter weekly.

In Charts 1 and 2 are found the growth curves of males and females, respectively, on a diet of fresh cow's milk with the various additions that were later made. It was somewhat surprising to find rats making such satisfactory initial growth on cow's milk considering the differences in composition and concentration between cow's and rat's milk (26). The later growth, however, was unsatisfactory both in males and females. Between the 50th and 100th day of life, especially in females, there was a decided retardation of growth, sometimes a considerable loss in weight, after which growth might continue but at a slower rate and on a much lower level. The animals on fresh milk failed to reproduce although there was satisfactory reproduction in animals from the same litters placed on stock rat food. The records of milk consumption are summarized in Tables I and II. This summary does not include all the animals because through regrouping and remating the record of a given pair was necessarily closed. Only those figures are given, therefore, which cover a substantially unbroken period, beginning at the time of weaning when the animals were placed on the experimental ration. In general the food consumption runs somewhat parallel with the rate of growth and a comparison of the fresh and dry milk figures shows that the amount of liquid which had to be ingested in fresh milk tended to diminish the consumption of milk solids in that form. Most of the fresh milk rats did not always consume milk to their capacity judging from the larger amounts of which they disposed in some periods. Perhaps the factors of humidity and temperature had some effect but of these we have no record. For the peculiar behavior of Rats 3025, 3026, 3015, and 3016 when they were regrouped and mated there seems no explanation. Food consumption fell appreciably after the mating, the rate of growth of the females was permanently slower, and the males, after a decline in weight for several weeks, renewed their growth somewhat but on a lower level. Of particular interest in this connection are the results recently

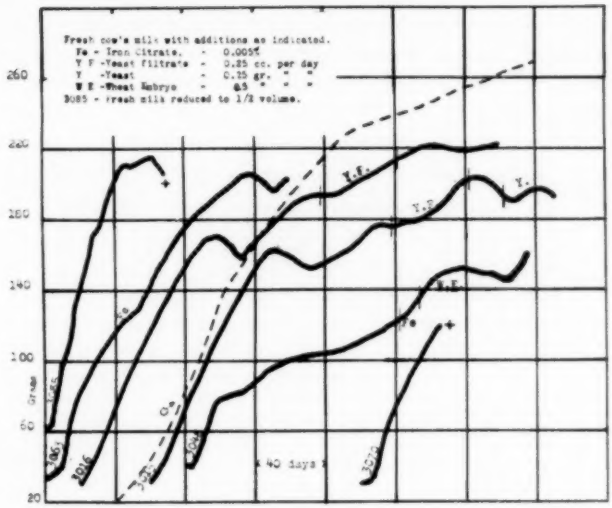

CHART 1.

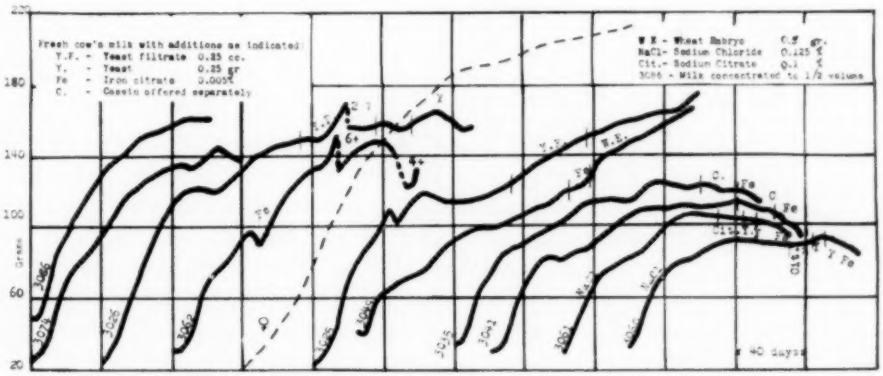

CHART 2.

TABLE I.

Food Intake of Rats on Fresh Milk.

The food intake is given in terms of solids (one-eighth of the volume of milk consumed) and the figures represent the average daily ingestion of two rats over 10 day periods beginning at the time of weaning. Unless otherwise indicated the animals were mated.

Nos. 3060, 3061.	Nos. 3062, 3063.	Nos. 3048, 3049.	Nos. 3025♀, 3026♀.	Nos. 3015♂, 3016♂.	Nos. 3035♀, 3041♀.	Nos. 3027♀, 3028♀.*	Nos. 3085, 3086.†
7.0	6.3	5.6	6.8	6.4	6.3	5.0	9.4
8.6	10.7	8.1	10.3	10.9	9.3	9.0	14.2
7.4	11.1	7.7	12.6	13.2	10.0	9.0	17.9
7.7	10.0	8.0	12.6	12.9	8.3	10.5	16.9
8.3	10.4	8.4		13.6	8.1	9.8	16.8
7.4	11.3	8.7		14.7	8.7	9.5	14.4
			Mated.				
			Nos. 3025, 3016.	Nos. 3015, 3026.			
7.4	13.2	8.2	10.2	12.1	8.2	11.8	13.0
6.1	11.8	7.7	10.2	10.2	8.0	13.2	
5.9	13.4	6.9	10.6	10.5		13.7	
5.7	13.0	7.9	12.0	11.7		13.2	
5.7	12.7	7.9	12.8	12.2		12.6	
5.8	12.0	8.3	11.2	11.4		13.8	
5.0	8.2	8.3	10.5	11.0		14.1	
	14.0	10.5	11.8	13.3		14.1	
		10.4	11.3	10.9		14.4	
		8.8	11.8	10.8		13.5	
		9.5	12.0	12.5		13.3	
		9.7	11.1	12.3		13.5	
			12.2	11.8		15.3‡	
			12.2	10.3		16.2	
			11.6	10.4		16.7	
				10.5		21.0§	
				8.8		24.0	
				9.0			

* Dry milk with water to equal fresh milk.

† Fresh milk concentrated to half its volume at low temperature.

‡ One-half the amount of water.

§ No water.

reported by Sherman and his coworkers (27). On a bread and milk ration in which milk furnished one-fifth of the total calories young rats made normal growth but the females usually failed to breed and no young were raised. When ground whole wheat replaced white bread or patent flour young were successfully suckled at the cost of considerable loss in weight of the mother and grew at less than normal rate. When the amount of milk was increased so as to supply two-fifths of the total calories and the rest by ground whole wheat, young were suckled without

TABLE II.

Food Intake of Rats on Dry Milk and Dry Milk Foods.

The figures represent the average daily ingestion of two rats over 10 day periods beginning at the time of weaning. The animals were mated unless otherwise indicated. Rat 3018 was alone.

No. 3018♂.	Nos. 3019♂, 3020♂.	Nos. 3069, 3073.	Nos. 3070, 3074.	Nos. 3056, 3057.	Nos. 3064♂, 3066♂.	Nos. 3067, 3071.	Nos. 3068, 3072.
3.5(?)	6.5(?)	7.8	6.1	7.7	9.8	8.7	7.7
4.4	10.2	11.9	8.8	7.9	13.5	13.6	11.1
5.9	11.6	12.8	9.0	8.6	17.4	16.4	13.4
6.1	15.3	14.0	10.2	10.4	19.3	15.4	14.5
6.5	13.8	14.0	9.7	7.7	15.7	15.0	15.0
6.9	14.6			6.5	16.8	18.9	15.4
7.6	18.2			6.2	16.4	18.2	15.3
6.4	20.2			3.0	16.5	19.2	16.7
7.5	11.6				17.7	18.7	19.0
9.7	14.9				18.0		19.4
					11.2		

undue loss of weight on the part of the mother and showed a fully normal rate of growth. A preliminary inference from these results (which the authors did not make) that a ration in which milk supplied all the calories would show equally good or even better results is not justified by the behavior of our animals, and it is evident that ground whole wheat supplied something which milk does not contain in adequate amounts if at all. In contrast with these results is the experiment of McCollum (28), in which a pig taken at weaning was kept on a ration of cow's milk for a period of 17 months, during which time the animal grew and seemed to be properly nourished. At 13 months she pro-

duced eight living and two dead pigs and successfully brought the young to an average weight of 17 pounds. Some additional iron was furnished by the drinking water and by the rusted parts of milk cans.

In a search for the cause of the inadequacy of the fresh milk ration various additions were made. Since milk is not so rich in water-soluble vitamine (29) as the original experiments of Hopkins (11) seem to indicate, and inasmuch as an inadequate amount of this substance has been shown to lead to infertility (30), a small amount (0.5 cc.) of yeast filtrate (after autolysis) was daily added to the milk fed to each of two pairs of animals (Nos. 3025, 3016, 3015, 3026). Yeast proteins were thus supplied in addition to vitamine. This supplement slightly increased the rate of growth in the males temporarily, and one of the females (No. 3026) became pregnant. The size of the litter could not be determined since only one (dead) and part of another were found. Subsequent addition of 0.5 gm. of yeast to the daily ration of this pair was without permanent effect. The abundant supply of water-soluble vitamine in wheat embryo suggested its use as a supplement to milk and for these preliminary experiments the wheat embryo was merely ground and ether-extracted since the fat is toxic for rats (10, 31), and in this powdered form 1 gm. per day was stirred into the milk supplied to Rats 3048 and 3049. Aside from a slight increase in food consumption and a temporary improvement in the rate of growth of the female this addition has thus far not yielded any positive results. Among many other procedures to be tried in connection with the problem of water-soluble vitamine is the addition to milk of further amounts of the same vitamine that it contains, and of preparations of the vitamine from other sources containing as little as possible of other ingredients.

Since milk has a low iron content, the addition of proportionate amounts of a solution of iron citrate[2] was made in several cases, in Rats 3062 and 3063 from the very beginning. The male made an excellent growth record for 120 days, losing thereafter; the female became pregnant and cast a litter of six which were all dead on the following day. Later she cast another litter of four

[2] 0.5 cc. of a 10 per cent solution per 100 cc. of milk.

which had disappeared by the next day. The growth of this pair was by far the best of any on raw milk and their milk consumption was generally somewhat above that of any others on raw milk. In certain of the animals as indicated in the charts additions of iron and of iron in conjunction with yeast were without effect. The addition of sodium either as chloride or citrate[3] and the supplying of casein fed separately were alike ineffective. The animals receiving a salt addition showed an especially low food intake and correspondingly poor growth.

To throw further light on whether the concentration of nutrients in milk was too small to supply adequate food in the volume that a rat could consume, two animals, Nos. 3085 and 3086, were fed on fresh milk concentrated to half its volume by evaporation under diminished pressure below 50°C. The male made an exceptional growth but died after a very small loss in weight from causes which autopsy did not reveal. The growth of the female was not very different from that of those on ordinary milk, but although a male that had been on stock food was later mated with her no pregnancy resulted within 26 days. The food intake of these animals in milk solids was comparable to the intake of dry milk in other animals.

The results obtained with dry milk are shown in Chart 3, and some of the food consumption records in Table II. Both males and females show a much more satisfactory growth than on fresh milk, the males being practically normal, the females somewhat below normal after about 75 days. None of the curves shows so pronounced a slowing of growth at 50 to 100 days as do those of the animals on fresh milk. The short period of decline in weight of Rats 3018 and 3019 with subsequent partial recovery accompanied a decrease in food consumption which in the case of No. 3019 at least can be ascribed to intestinal infection, with diarrhea. This condition was not observed with No. 3018, and No. 3036, which was mated with No. 3018 at that time, showed no decline in weight. These growth results are in partial agreement with earlier experience. Lane-Claypon (32) found that rats showed a slightly more rapid gain in weight on dry milk than upon boiled milk and these in turn a rather more rapid gain

[3] 1 cc. of a 12.5 per cent solution per 100 cc. of milk.

than those fed on raw milk. However, bread was a part of the ration. Winfield (33) fed rats on dry milk exclusively and found that growth in both sexes fell below normal when one-half to two-thirds of the adult weight was reached and was only renewed when a mixed dietary was put in place of dry milk. There are no data on reproduction. The considerably better growth performance of rats on dried milk as compared with fresh milk is primarily a result of their getting more food. The limitation which liquid milk placed on food consumption is shown

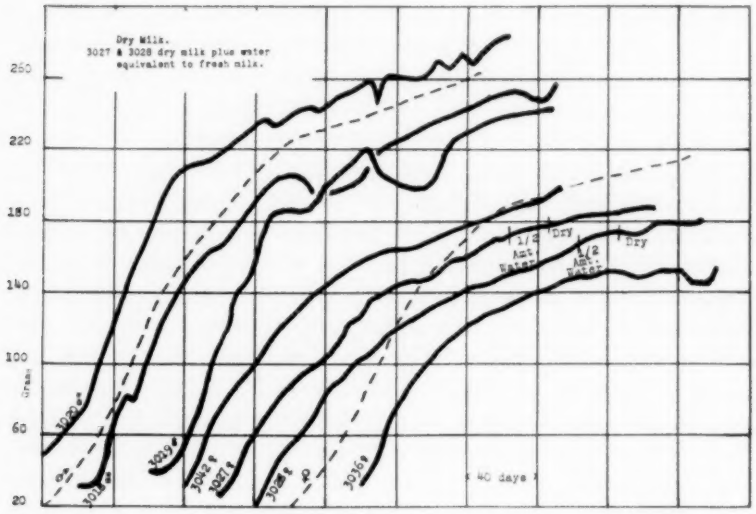

CHART 3.

in the food record of Rats 3027 and 3028 (Table I) which were fed dry milk moistened with seven times its weight of water so as to approximate the composition of fresh milk. While their food intake in total solids was higher than that of animals on fresh milk it increased when the amount of water was halved and almost doubled when the water was omitted entirely. There was no reproduction among these rats on dry milk even though they were mated variously.

The retardation of growth between 50 and 100 days in females on dry milk, and a similar behavior of both males and females on

fresh milk, indicate the possibility of a requirement for new as well as for additional food. Rats arrive at sexual maturity at about the age of 60 days; the thymus undergoes atrophy and the gonads especially in the female develop rapidly during the next 50 days. If we employ the terminology of growth cycles as developed by Robertson, this age in the rat falls in the early portion of the third growth cycle, the beginning of which he has found for man (34) and for mice (35) to precede the age of puberty. The transition from one cycle to another he considers a critical period and in his own and other investigations various substances have been shown to influence certain types of growth differently. The results of milk feeding in rats, especially in the

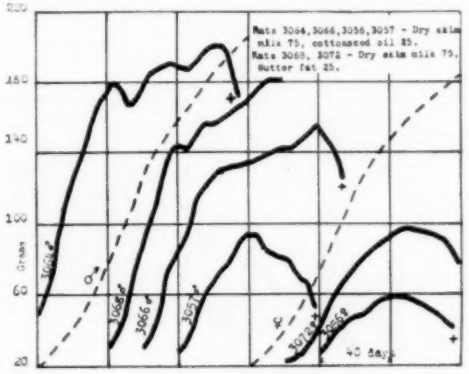

CHART 4.

female, suggest a qualitative as well as a quantitative inadequacy of milk to meet the demands of the third growth cycle. It is not impossible that milk might contain substances inhibitory to growth in the third cycle.

Among the modifications of the dry milk ration which it was thought might throw some light on the question were those involving fat and fat-soluble vitamine. If the latter had been partially destroyed, sufficiently perhaps to affect reproduction, then the use of skim milk powder to which an equivalent amount of butter fat was added should be more satisfactory. The animals on this ration, Nos. 3068 and 3072, Chart 4, did no better than others on dry whole milk, the female especially making very

poor growth. Several animals were also placed on a ration of dry skim milk 75 per cent and cotton-seed oil 25 per cent. Three males, Nos. 3057, 3064, and 3066, Chart 4, made satisfactory growth for 40 to 50 days, at which point all the curves "break" and while two of them continued to grow slowly they all died in 80 to 125 days. The record of the female, No. 3056, Chart 4, on this ration is very poor. The content of this ration in butter fat is about 1 per cent and from the figures of food intake of the two groups, Nos. 3064, 3066 and 3056, 3057, it is seen that none of the four animals ever obtained 0.1 gm. of butter fat per day, an amount on which Osborne and Mendel have recently reported (36) good growth and maintenance for about 240 days. It is

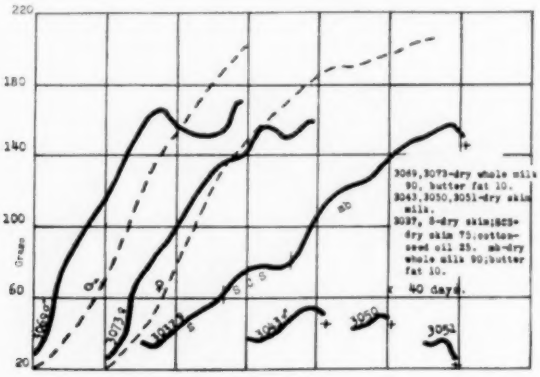

Chart 5.

probable that these four animals succumbed because of lack of fat-soluble vitamine although there were never signs of xerophthalmia (37) and aside from emaciation only roughened coats. On skim milk powder alone rats succumb quickly following little or no growth (Rats 3043, 3050, 3051, Chart 5), an effect which, in the light of our results, could be ascribed in part at least to a deprivation of fat aside from a lack of fat-soluble vitamine.

The effect of an increase in the amount of fat and fat-soluble vitamine was also determined by feeding a ration containing dry milk 90 per cent and butter fat 10 per cent. The animals on this ration, Nos. 3069 and 3073, Chart 5, made good growth for about 80 days and the female cast a litter the size of which could not be

recorded, since only one (dead) was found; at autopsy the uterus showed eight placental scars but the mother's weight had not been affected so far as the weekly weighings showed. Both curves broke after 70 to 90 days of this ration. Another animal, No. 3037, Chart 5, which had been transferred from skim milk powder to the same plus 25 per cent of cotton-seed oil with continued very slow growth, was placed on whole milk powder plus 10 per cent butter fat. Growth was resumed at a normal rate on the lower level but this animal died after about 100 days on this ration.

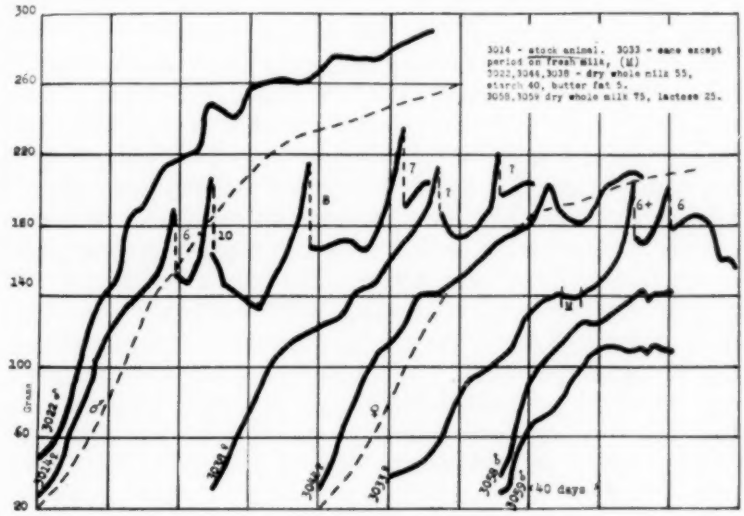

CHART 6.

A further modification of the dry whole milk ration gave interesting results. This consisted of dry milk 55, starch 40, and butter fat 5 per cent, and upon it three rats (Nos. 3022, 3038, and 3044, Chart 6) made entirely satisfactory growth over the whole period of the experiment. The curves of the females do not show the marked break at 50 to 100 days which many on milk alone exhibit. One of the females cast two litters but only one of the young animals was found. The weight curve of the other suggests a litter but none was found. As this ration contains only 55 per cent of the protein, salts, and water-soluble

vitamine of dry milk and about 70 per cent of its fat content, it is somewhat analagous to the milk food[4] used by Osborne and Mendel (17) on which they reported entirely normal growth and reproduction. However, in their charts[5] it is seen that in the two animals discussed reproduction did not occur until relatively late, in one at about 400 days of age, close to menopause, in the other at 185 days of age, while breeding should normally begin at 90 to 100 days. It is not stated whether these animals were mated throughout the period of observation. Without exception the females of our small colony on stock food have had their first litters when under 125 days of age and the curve of No. 3014, Chart 6, shows typical behavior in this respect. A food can hardly be considered entirely successful when reproduction is delayed beyond the normal age at which it should begin, but it is extremely interesting that animals are able to reproduce on a 60 per cent dry milk ration with lard and starch when they do not do so on dry milk alone. In contrast with our less successful animals on 55 per cent milk powder with starch and butter fat are two others, Nos. 3058 and 3059, Chart 6, which were placed on a ration of 75 per cent dry milk and 25 per cent lactose. In this ration the proteins, salts, fats, and vitamines are more abundant than in the other, the principal difference being a substitution of lactose for starch. But these rats (males) stopped growing, one at 112 gm. the other at 143 gm., and this despite the fact that lactose occupies a unique position among carbohydrates, while starch is not believed to carry any vitamines (38) or to possess any peculiar virtues. Our data are as yet too limited even to justify hypothesis but the results of Osborne and Mendel just quoted as well as those of Sherman mentioned above point in the same direction. A tentative explanation would be that milk possesses an inhibitory agent for adolescent growth and reproduction, the effect of which is partially obviated by dilution with other foodstuffs and that these foodstuffs, in particular starch (and lard was also used by Osborne and Mendel), provide a requisite which milk does not possess. What these substances might be is a matter of conjecture, but starch contains phosphorus in organic combination with a carbohydrate the nature of which is unknown (39, 40).

[4] Whole milk powder 60, starch 12, lard 28 per cent.

[5] Osborne and Mendel (17), p. 320.

The possibility of transient or permanent infection of the lungs, alimentary tract, or other organs, with a resultant subnormal efficiency, is a very real one especially when the food is inadequate. The susceptibility of rats to lung disease militates somewhat against their value as a laboratory animal, but in our experience the presence of this infection is not always evidenced by the animal's capacity for growth and reproduction. Thus Rat 3014 whose curve is shown in Chart 6 had very bad lungs, which probably accounts for her low weight, though her reproduction record is fairly satisfactory. Similar discrepancies are shown in Table III. According to McCollum[6] rats on a milk diet have foul cages, a fact not entirely borne out by our experience, perhaps because our animals were on an exclusively milk diet from weaning. The cages of animals on fresh milk required more frequent renewal than others but except in a few cases of diarrhea the feces were well formed and without abnormal odor.

Several attempts have been made to determine whether the incapacity for reproduction on milk rations resides in the male or the female, but the introduction for limited periods of males or females from stock food into cages with animals on milk has not given any positive results. Rat 3033, Chart 6, may be cited. From stock food without previous mating she was transferred at the age of 125 days to a cage with a male on raw milk. After 11 days she was returned to a stock cage and mated. 30 days thereafter she cast a litter of six which she did not raise. Impregnation had evidently not been accomplished by the milk-fed male but after her return to the stock cage. A second litter followed immediately (after 3 weeks) which she raised successfully, but the choice of this animal for a test was perhaps unfortunate because of some pathological condition evidenced by her subsequent decline in weight. Her ovaries were found to be below normal in weight (Table III).

Many of the animals whose curves are given have not been continued on experiment since it was desired to examine them post mortem. In Table III are given the weights of the sex glands together with brief autopsy findings. The weights of the thymus would also have been very desirable but it was not possible to

[6] McCollum (28), p. 76.

TABLE III.

No. of animal.	Age.	Final weight.	Weight of gonads.	Normal weight of gonads.*	Difference.	Ration.	Autopsy.	
							State of lungs.†	Remarks.
	days	*gm.*	*gm.*	*gm.*	*per cent*			
Males.								
3001	367	222	2.17	2.30	− 5.7	Stock.		Small intestine somewhat inflamed.
3017	271	212	2.56	2.25	+ 6.8	"	++	
3046	206	250	2.45	2.45	± 0.0	"	+	
3015	262	177	2.45	2.02	+21.3	Raw milk.	+++	Abundant fat.
3016	269	229	2.16	2.33	− 7.3	" "	+++	
3019	255	239	2.80	2.40	+16.7	Dry "	+	Fat. Fine condition.
3022	257	292	2.29	2.64	−13.3	55 per cent dry milk.	+	Fat very abundant.
3045	201	136	1.82	1.70	+ 7.1	Raw milk.	++	
3058	169	159	2.04	1.88	+ 8.5	Dry " + lactose.	+	
3059	169	85	1.03	1.08	− 4.6	" " + "	++	Emaciated.
3063	164	205	2.14	2.21	− 3.2	Raw " + Fe.		
3064	144	171	1.05	1.99	−47.2	Skim " powder + cotton-seed oil.	+++	
3066	140	125	1.65	1.58	+ 4.4	" " " + " "	++	
3068	129	187	1.98	2.10	− 5.7	" " " + butter fat.		
3069	145	170	2.26	1.98	+14.1	Whole " " + " "		
Females.								
3006	374	200	0.071‡	0.049	+44.9	Stock.	++	
3014	276	192	0.065‡	0.049	+32.6	"	+++	
3029	268	205	0.051‡	0.049	+ 4.1	"		Fat abundant.
3033	223	155	0.023‡	0.047	−51.1	"	+	

* Donaldson (41), p. 101.

† State of lungs: fair +, bad ++, very bad +++. Autopsy findings normal unless otherwise indicated.

‡ Animals had been pregnant at least once.

3025	245	175	0.012	0.048	−75.0	Raw milk.	+	Liver slightly hemorrhagic.
3026	238	157	0.036‡	0.047	−23.4	" "		Abundant fat.
3035	204	106	0.015	0.032	−53.1	" "	++	
3038	216	193	0.038‡	0.049	−22.4	55 per cent dry milk.	+	Liver hemorrhagic; uterus inflamed.
3041	201	93	0.015	0.022	−31.8	Raw milk.		
3044	213	201	0.026‡	0.049	−46.9	55 per cent dry milk.	+	Abundant fat; intestine and uterus inflamed.
3060	155	83	0.014	0.016	−12.5	Raw milk + NaCl.		
3061	155	94	0.012	0.024	−50.0	" " + NaCl.		
3062	164	134	0.037‡	0.045	−17.8	" " + Fe.	+	Uterus inflamed.
3072	126	74	0.016	0.013	+23.1	Skim " powder + butter fat.	++	
3073	142	160	0.047	0.048	− 2.1	Whole " " + " "		
3074	154	140	0.026	0.046	−43.5	Raw "		
3086	135	153	0.030	0.047	−36.2	Concentrated milk.	+	

obtain them at this time. The sex glands were dissected and weighed as indicated by Donaldson (41), but without using a moist chamber, and the secretion of the male gonads was usually also examined under the microscope. With one exception the weights of the testes were within normal limits; sperm were always present and if the examination was made immediately after the death of the animal they were seen to be motile. The ovaries, on the other hand, showed marked variation and with one exception the ovary weights of the animals on milk rations were below normal, even 50 per cent and more. The conclusion seems

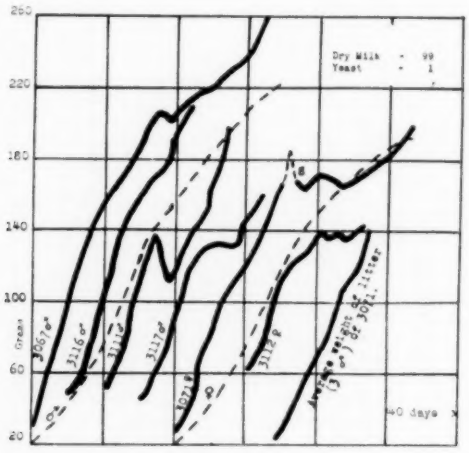

CHART 7.

justified, therefore, that an exclusively milk ration fails to supply something which is specifically required for reproduction in the female, being at the same time adequate, in so far as sperm production goes, for the male. Further breeding trials must be made to determine the complete efficiency of the male.

One further series of animals should be mentioned. The observations in experiments being conducted on mice that an addition of yeast to a dry milk ration effected better reproduction than had been secured, as well as the partially successful result of yeast filtrate addition to fresh milk in rats (Chart 2), suggested the addition of dry yeast to whole milk powder. Rats 3067 and 3071, Chart 7, to which this was fed (1 per cent yeast) are

the only animals of the milk-fed series that have thus far not only made satisfactory growth themselves but also have successfully raised young. Of the original litter of eight, five (died (?) and) were eaten by the parents, but the three remaining have grown fairly well on the same ration as the composite curve indicates. There has not been another litter although the parents are still mated. The growth of other rats on this ration is shown in Chart 7 and that of rats on whole milk powder containing 5 per cent yeast in Chart 8. The performance of these animals may show whether yeast really supplies something unique. The results are at least interesting in comparison with those of Os-

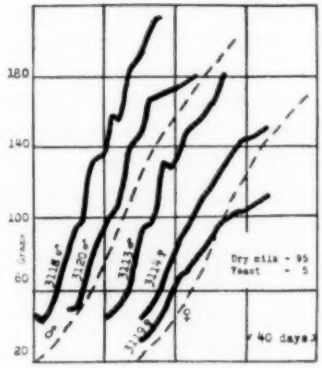

CHART 8.

borne and Mendel (42) on four males fed yeast as their sole source of protein and water-soluble vitamine, which grew normally but remained infertile.

A word should be said with reference to the factor of variability. Since all these experimental animals were taken from weaning, the usual period during which they are allowed to grow to a weight of 50 or 60 gm. before being placed on an experimental ration is omitted. In this period it is possible to detect subnormality more readily than in the earlier stage before weaning. While we used no animals that were subnormal it is quite possible for the above reason that a larger number of animals will be required to establish these results than would otherwise be necessary. It is safe to say that variability is in general too little considered in this type of investigation.

SUMMARY.

In these preliminary experiments young rats taken at weaning were placed on various rations consisting primarily of cow's milk, fresh and desiccated. On fresh milk rats made good initial growth but beginning between the 50th and 100th day of life, especially in the females, there was a decided retardation. There was no reproduction. The addition of yeast filtrate temporarily increased the rate of growth and one female became pregnant, but the litter was eaten. Addition of wheat embryo also increased the rate of growth with no further effect thus far. When milk was supplemented with iron citrate growth was much more satisfactory but reproduction was not successful. The growth failure was in part the result of the dilute form of the food, for on dry milk rats made much better growth and their average food consumption in milk solids was considerably greater than that of those fed on fresh milk. The females, however, remained somewhat below normal after 75 days and again there was no reproduction. Neither substitution of dry skim milk to which butter fat was added equivalent to that in dry whole milk nor the addition of 10 per cent of butter fat to dry whole milk was successful.

On a ration containing 55 per cent of dry whole milk, 40 per cent of starch, and 5 per cent of butter fat, both male and female rats made practically normal growth, and the females bore young but did not raise them. A similar ration has been used by other investigators who secured not only satisfactory growth but also reproduction, while other investigators have, like ourselves, not had success with whole dry milk alone. Dilution of dry milk by 25 per cent lactose resulted in poor growth and no reproduction. The gonads of many of the animals were weighed; the testes were with one exception of normal weight and contained motile sperm; the ovaries on the other hand were much under normal weight, even 50 per cent and more.

Possibly milk is lacking both quantitatively and qualitatively in substances necessary for successful adolescent growth and reproduction, especially in the female, and it may contain substances that are inhibitory to the growth of an animal in the third or mature growth cycle. A ration containing dry milk and 1 per cent of yeast is the only one on which normal growth and par-

tially successful reproduction were obtained, and the growth of rats on this ration and on one containing 5 per cent of yeast is thus far practically normal. Whether yeast supplies something unique remains to be seen. The factor of variability necessitates data on a larger number of animals before definite conclusions can be drawn.

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THE EFFECT OF COOKING ON THE WATER-SOLUBLE VITAMINE IN CARROTS AND NAVY BEANS.

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The increasing use of highly refined food products, in which the content of vitamine is low, makes correspondingly great the necessity of including, in the diet, foods which are especially rich in these indispensable factors. There is considerable evidence, derived from animal feeding experiments, to show that some of the common vegetables are valuable as sources of the water-soluble vitamine. Among those that have been found to contain relatively large amounts of this substance are cabbage,[1, 2] spinach,[2] potato,[3] navy bean,[4] carrot,[5] and onion, turnip, beet, and tomato.[6] But in most of these experiments the uncooked vegetables, fresh or dried, have been used. It has been found that in cooking there is a considerable loss of protein and other constituents by solution in the cooking water. The vitamine content may also be diminished to such an extent that the cooked and uncooked vegetables cannot be compared as sources of this substance. It is therefore desirable to obtain information concerning the effect of the various processes of preparation on this important constituent. In the experiments reported here the

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[1] McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187.

[3] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1918, xxxvi, 197.

[4] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.

[5] Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 171. Denton, M. C., and Kohman, E., *J. Biol. Chem.*, 1918, xxxvi, 249.

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxix, 29.

vitamine was determined in carrots and navy beans cooked by methods commonly used in the household.

Quantitative determinations of the water-soluble vitamine in foods have been made either by means of growth experiments on animals or experiments for testing the antineuritic properties, as measured by the preventive or curative effect. Most investigators consider that the water-soluble, growth-promoting vitamine and the antineuritic substance are identical. Results obtained from both methods of experimentation have been reported to show the effect of high temperatures on the water-soluble vitamine in food. These results, however, show considerable variance.

Most of the evidence agrees that the vitamine is not destroyed by heating for considerable periods of time at temperatures of 100°C. or lower. McCollum and Davis[7] found that wheat embryo, milk whey, and crude lactose from which the vitamine was not entirely removed could be heated for 1 hour at 15 pounds pressure (120°C.) without apparent loss of the growth-promoting property. McCollum, Simmonds, and Pitz[4] detected little, if any, diminution when navy beans were heated in a moist condition at 120°C. for $1\frac{1}{4}$ hours.

Daniels and McClurg[8] conclude from results obtained in feeding young rats that cooking cabbage and beans (navy and soy) did not destroy the water-soluble vitamine to any great degree. They cooked the beans in three ways—in water at 100°C., in dilute alkali at 100°, and at 120° for 15 to 20 minutes. However, the diets fed contained for every 100 gm. of dry food the equivalents of 50 gm. of navy beans, or 55 gm. of soy beans, or 250 gm. of fresh cabbage which undoubtedly supplied an excess of the growth-promoting vitamine. McCollum, Simmonds, and Pitz[4] found that 25 gm. of navy beans for 100 gm. of dry food was sufficient to supply this dietary essential. The vitamine content might have been reduced to 50 per cent of the original value and the loss not detected in these experiments.

Chick and Hume,[9] on the other hand, report that heating wheat germ at a mean temperature of 113°C. for 40 minutes

[7] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

[8] Daniels, A. L., and McClurg, N. I., *J. Biol. Chem.*, 1919, xxxvii, 201.

[9] Chick, H., and Hume, E. M., *Proc. Roy. Soc. London, Series B*, 1917, xc, 60.

reduced its curative effect, when fed to polyneuritic pigeons, to one-half the original amount. Heating the wheat germ at 118–124°C. for 2 hours caused such rapid destruction that its antineuritic activity was less than one-fourth that of the untreated material. There was no significant decrease by heating at 100°C. for 2 hours.

Davis[10] finds that the ordinary pigeon ration of corn, Kaffir corn, barley, and split peas when heated for 2½ hours at 120°C. no longer protects against polyneuritis. In fact the mixture treated thus is used as the beri-beri-producing ration.

There is a similar lack of agreement in the evidence concerning the stability of the water-soluble vitamine to alkali. McCollum and Simmonds[11] report almost complete destruction when an extract of navy beans was made alkaline to 0.28 per cent with sodium hydroxide and heated under a reflux for 1 hour. Daniels and McClurg,[8] on the contrary, found no apparent loss when an alkaline water extract of beans was heated under the same conditions. However, the material used by McCollum was extracted from the beans with alcohol, after a preliminary extraction with ether. There is evidence which indicates that the vitamine exists naturally in some protective combination which is broken up by the treatment with ether and extraction with alcohol.[12]

Osborne, Wakeman, and Ferry[13] digested brewery yeast with ten times its weight of 0.1 N sodium hydroxide solution for 21½ hours and then heated it on the water bath for 2 hours. After this treatment it still produced as prompt recovery when fed to rats that had begun to lose weight on a diet practically vitamine-free as an equivalent amount of untreated yeast. They conclude that the water-soluble vitamine is more stable to alkali than has usually been supposed. But in this case also the yeast was not subjected to extraction with ether before heating with the alkali.

[10] Davis, M., *J. Home Econ.*, 1920, xii, 206.

[11] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

[12] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

[13] Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

A much simpler and more delicate method for the quantitative determination of the water-soluble vitamine has been recently suggested by Williams.[12] He found that yeast cells require for their growth a substance which he concludes from evidence he has obtained is identical with the substance which in animal nutrition prevents polyneuritis. He showed that the rate of reproduction of a single yeast cell paralleled, within limits, the concentration of the vitamine which was added to the medium in which the yeast was grown. Bachmann[14] also found that certain yeasts would not ferment unless some substance rich in water-soluble vitamine was added to a medium containing inorganic salts and sugar. She also suggested that such yeast might be used to determine quantitatively the amounts of vitamine in various substances.

Since there is such apparent disagreement in the results reported by different investigators as to the effect on the vitamine content of subjecting foods to ordinary cooking processes, it seemed advisable to carry out experiments using the yeast method. Two vegetables, carrots and navy beans, were cooked by the methods most commonly used and the vitamine in the cooked vegetable and in the cooking water was determined separately. Carrots were cooked at 100°C. for 30 minutes and canned at 10 pounds pressure for 45 minutes. For comparison the vitamine content of the raw carrots was also found. Since dried legumes require a longer cooking period than other vegetables, experiments were carried out with navy beans. The beans were cooked by three methods: in distilled water at 100°C. for 1½ hours, in 0.5 per cent sodium bicarbonate solution for 1 hour and 10 minutes, and in distilled water at 120° for 30 minutes.

EXPERIMENTAL.

Method of Determining the Vitamine.—A quantitative method of determining the amount of vitamine in a solution was perfected by Williams[12] and Swoboda.[15] The average growth of single yeast cells, when different volumes of a vitamine solution were added to the medium in which the yeast was grown, was

[14] Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

[15] Swoboda, F. K., unpublished experiment.

found to have a definite quantitative relation to the amount of vitamine added. The best comparative results were obtained when the growth was counted at the end of 18 hours. When no vitamine was added to the medium an average growth of four to eight cells was obtained from a single cell. Since this method has been given in detail by Williams[12] and Swoboda[15] it will be described only briefly here.

The medium used for the growth of the yeast contained the following substances dissolved in 1 liter of redistilled water.

20 gm. of sucrose (Kahlbaum).
3 " " $(NH_4)_2SO_4$ (Merck).
2 " " KH_2PO_4 (Merck).
1.5 " " asparagine (Kahlbaum).
0.25 " " $CaCl_2$ (Central Scientific).
0.25 " " $MgSO_4$ (Baker).

25 cc. portions of this solution were measured into flasks and autoclaved at 10 pounds pressure. After cooling, 3 and 5 cc. quantities of the solution to be tested were added to the medium and when necessary redistilled water to make up the volume to 30 cc. Duplicate solutions were prepared in each case and blanks run for controls. Finally 1 cc. of a suspension of Fleischmann's yeast in redistilled water was added to each solution. This suspension was made by shaking a piece of yeast about the size of a small grain of wheat in 30 cc. of water. Hanging drops were then made from each solution with a sterile pen.

The slides were examined immediately to count the single cells. Only those drops which contained three or less than three single cells were considered. The slides were then put into an incubator kept at 30°C. A second reading was made at the end of 6 hours to verify the first record and to detect any cells which did not grow. Finally a count was made of the cells in each drop at the end of 18 hours.

Preparation of Vitamine Solution from the Vegetables.—In using the yeast method the vitamine must be in solution. It was necessary therefore to extract the vitamine from the vegetable quantitatively. Dixon and Atkins[16] found that the plant juices

[16] Dixon, H. H., and Atkins, W. R. G., *Sc. Proc. Roy. Dublin Soc.*, 1912–13, xiii, 422. Gortner, R. A., Lawrence, J. V., and Harris, J. A., *Biochem. Bull.*, 1916, v, 139.

could be removed from the tissue most effectively by freezing the material and then pressing. This method was adopted for obtaining the juice from raw carrots. To make certain that freezing has no effect on the vitamine a yeast extract of known

TABLE I.

Effect of Freezing on the Vitamine.

	Quantity added.	No. of cells growing.	Maximum.	Minimum	Average.	Average of duplicate determinations.
	cc.					
Before freezing.....	3	20	170	40	126	
" "	3	18	192	30	142	134
After "	3	14	160	80	128	
" "	3	20	200	80	132	130

TABLE II.

Lack of Adsorption of Vitamine by Berkefeld Filter.

	Quantity added.	No. of cells growing.	Maximum.	Minimum.	Average.	Average of duplicate determinations.
	cc.					
Dilute solution.						
Unfiltered.....	3	17	71	22	37.7	
	3	21	60	23	33	35.3
Filtered.....	3	25	56	25	35	
	3	16	60	16	36	35.5
Concentrated solution.						
Unfiltered.....	5	16	352	130	218	
	5	23	400	150	226	222
Filtered.....	5	18	400	112	215	
	5	14	400	120	212	213

activity was frozen and kept at $-7°$C. for 2 weeks. The results given in Table I show that there was no loss in activity caused by this treatment.

It is also necessary that the vitamine solution should be sterile since other organisms introduced into the medium interfere with the growth of the yeast. To avoid sterilization by heat the vita-

mine solutions prepared from the vegetables were run through a Berkefeld filter. Tests were made with yeast extracts of different concentrations which had previously been sterilized by autoclaving to find if any of the vitamine was adsorbed by the filter. The results given in Table II show that there was no loss of the active substance by this treatment.

Preparation of Carrots.—Carrots were purchased from the market in the fall. They were mature but fresh and still retained some of the green stems. In scraping off the skin, care was taken to remove as little of the vegetable as possible. To obtain uniform samples for the separate experiments the carrots were cut lengthwise into thirds. One third of each carrot was then used as described below for the determination of vitamine in raw carrots, another third was cooked at 100°C., and the other portion was canned at 10 pounds pressure.

Extraction of Juice from Uncooked Carrots.—The carrots were ground in a meat chopper, packed in a metal cylinder, and frozen at −7°C. The solid block of vegetable was then shaved to a fine snow in a Kossel[17] hasher. After thorough mixing, two portions of this finely divided material were immediately weighed out for determination of moisture. A third portion to be used for the determination of vitamine was put into a tincture press and the juice expressed. In order to remove the vitamine from the vegetable quantitatively, the dry residue was covered with water, allowed to stand 15 minutes, and again pressed. This was repeated six times when the extraction proved to be complete. The fifth extract when tested gave an average growth of only three cells per gram of the original fresh carrot. A sixth extraction was made to assure complete removal of the vitamine.

The combined filtrate and washings from 45.5 gm. of carrots were diluted to 2 liters, filtered through a Berkefeld filter, and used for the determination of vitamine. It was necessary to dilute the solution sufficiently to keep the number of yeast cells produced in a single colony within a range that could be counted. When the growth was heavy a micrometer disk was used to aid in counting. The colonies sometimes grew in three dimensions. It was necessary then to make an estimation of the number of cells.

[17] Kossel, A., *Z. physiol. Chem.*, 1901, xxxiii, 5.

Carrots Cooked at 100°C.—A second portion of the fresh carrots was cut into ½ inch cubes, and 200 gm. were weighed out and cooked in distilled water for 30 minutes. The cooking water was drained off and diluted to 1 liter. The cooked carrots were mashed in a mortar and the juice was obtained by pressing as in the case of the frozen carrots. The juice and washings from the cooked carrots were diluted to 6 liters.

Canned Carrots.—326 gm. of the same lot of carrots were diced and packed in a pint jar. Boiling water was added to fill the jar and the vegetable was then subjected to 10 pounds pressure for 45 minutes. The water in the can was drained off and diluted to 3 liters. The entire 326 gm. were too bulky to handle conveniently in the press, so, after mashing the vegetable and mixing, 79 gm. (the equivalent of 74 gm. of raw carrots) were weighed out. The juice from this amount was expressed and diluted to 3 liters.

Navy Beans Cooked at 100°C.—30 gm. of navy beans were cooked in 150 cc. of distilled water for 1½ hours. The cooking water was diluted to 1,200 cc. It was found more difficult to remove the vitamine quantitatively from the beans than from the carrots. For each extraction the bean pulp was covered with water and warmed gently for at least 1 hour before pressing. Repeating this seven times finally extracted all the vitamine. The juice and washings from the 30 gm. of beans were diluted to 1,200 cc.

Navy Beans Cooked in Dilute Alkali.—30 gm. of navy beans were cooked in 120 cc. of 0.5 per cent sodium bicarbonate solution for 1 hour and 10 minutes. The excess of alkali in the cooking water was neutralized to phenolphthalein with 0.1 N phosphoric acid. It took just 3.4 cc. of acid for the entire 1,200 cc. of solution. The extract from the beans cooked in the bicarbonate solution was already acid to phenolphthalein. Most of the alkali was therefore neutralized by the natural acids of the vegetable.

Navy Beans Cooked at 120°C.—30 gm. of a new lot of beans were cooked in 150 cc. of distilled water under 15 pounds pressure for 30 minutes. These beans were very soft and considerably broken. This accounts for the fact that in this case 70 per cent of the vitamine was found in the cooking water.

For comparison 30 gm. of this same lot of beans were cooked at 100°C. for 1½ hours. The cooking water and the extract of the vegetable were in each case made up to 1,200 cc.

Discussion of the Method.

It will be seen by consulting the tables that the maximum and minimum growth from single cells in a given experiment shows considerable variation. However, the majority of the colonies were close to the average so that by inspection of the results an estimate could be made that was close to the actual average. For example, a slide in which the maximum growth was 96, the minimum 10, and the average 42, 17 out of the 26 colonies had from 35 to 50 cells.

It is not possible always to account for the unusually high or low values. Occasionally large, somewhat transparent cells were found which did not grow so vigorously as the smaller ones. When some of the cells in a drop died and became disintegrated the growth of the remaining cells was inhibited to some extent. There is also the possibility of minute particles of toxic material on the cover-slip which might check the growth. Merest traces of cleaning solution left on the cover-slip had an exceedingly toxic effect.

When proper precautions were taken, however, and when averages were made of a large number of colonies, the results showed a definite quantitative relation between the amount of vitamine added and the growth obtained. In the later work two slides were prepared from each solution making a total of 144 hanging drops for each concentration.

To illustrate how well the results check, it was found that when 3 cc. of a given vitamine solution were added to the medium the average growth from 74 colonies was 86; the average growth from 137 colonies for 5 cc. of the same solution was 136.3. Subtracting the blank which was 6.7 cells, the ratio of 79.3 to 129.6 is 3 to 4.9 instead of 3 to 5.

Two determinations were made on separate samples of raw carrots and of navy beans cooked at 100°C. The results for the carrots were 516 cells and 552 cells respectively, per gram of the fresh material, and for the beans 2,034 cells and 2,116 cells per

gram. Because such concordant results can be obtained this method is of value for quantitative as well as qualitative tests. Its use requires much less time and labor than do the feeding experiments ordinarily used.

DISCUSSION.

The data given in Tables III and IV show that both carrots and navy beans are rich in water-soluble vitamine. The value obtained for dry yeast (Fleischmann's, starch-free) which is very high in water-soluble vitamine is 28,000 cells per gram. Carrots, dry weight, have a value about one-sixth as great and navy beans about one-twelfth. Osborne and Mendel[2] found that it required about five times as much dried spinach to produce satisfactory growth in young rats as it did of dried brewery yeast. Carrots, then, compare favorably with spinach as a source of water-soluble vitamine since brewery yeast contains less of this constituent than the pure Fleischmann's yeast. These results also concord with those previously reported for carrots by Sugiura and Benedict and by Denton and Kohman[3] and for navy beans by McCollum, Simmonds, and Pitz.[4] It also furnishes additional evidence as to the identity of the water-soluble vitamine and the yeast growth-stimulating substance.

Cooking carrots at 100°C. for 30 minutes caused no loss of the water-soluble vitamine. In the case of beans no comparison was made with the uncooked vegetable, but it is probable that there is no significant loss even in the longer period of cooking.

The beans cooked at the higher temperature, 120°C. for 30 minutes, showed a decided decrease in activity, a loss of 40.6 per cent. This agrees with the results of Chick and Hume[9] and of Davis.[10] There was probably a maximum loss in this experiment. It would take only a short time for the entire mass to become heated because of the small bulk. The beans were therefore subjected to the high temperature for almost the full period of heating. Then too, a very large proportion of the vitamine, 70 per cent, was in the cooking water due to the fact that the beans were overcooked and somewhat broken. The vitamine is probably less stable in solution than when it is retained in the vegetable tissue. An extract of beans heated at 115°C. for 10 minutes showed a loss of 24.4 per cent of the active substances.

TABLE III.

Quantity added.	No. of cells growing.	Maximum.	Minimum.	Average.	Average of duplicate determinations.	No. of cells for 1 gm. of original material, corrected for blank.		
						Fresh weight.	Average.	Dry weight = 11.32% of fresh weight.
A. Raw carrots. Extract from 45.5 gm. of fresh raw carrots diluted to 2 liters.								
cc.								
None.	31	17	3	7.3	7.0			
"	27	14	5	6.6				
3	26	96	10	41.8	42.9	525		
3	19	96	16	44.1				
5	26	101	20	62.2	64.8			
5	26	160	16	67.4		508	516	4,558
B. Cooked carrots. Extract from 200 gm. of cooked carrots diluted to 6 liters.								
None.	29	8	2	4.6				
"	39	7	2	3.5	4.1			
3	20	62	6	25.5				
3	13	59	12	29.5	27.5	234		
5	24	100	15	50.7	50.7	279	256	2,261
Cooking water from 200 gm. of carrots diluted to 1 liter.								
None	21	12	3	6.8	6.8			
3	26	330	126	175.5				
3	33	255	80	185.9	180.5	289		
5	23	600	125	249.5				
5	22	450	165	280.4	265.0	258	273	2,428
Total for cooked carrots.....							529	4,689
C. Canned carrots. Extract from 74 gm. of canned carrots diluted to 3 liters.								
None.	32	12	3	7.0				
"	26	15	2	7.8	7.4			
3	21	76	8	36.8				
3	27	50	14	32.5	34.6	367		
5	18	95	30	51.4				
5	14	104	20	56.8	54.1	378	373	3,304
Water from 326 gm. of canned carrots diluted to 3 liters.								
3	24	105	40	65.7				
3	25	150	48	71.6	68.6	188		
5	30	250	75	133.2	133.2	233	210	1,855
Total for canned carrots.....							583	5,159

TABLE IV.

Quantity added.	No. of cells growing.	Maximum.	Minimum.	Average.	Average of duplicate determinations.	No. of cells for 1 gm. of original material, corrected for blank.	
							Average.
A. Beans cooked at 100°C.							
cc.							
None.	32	12	3	6 0			
"	24	9	2	5.6	5.8		
Extract from 30 gm. of beans.							
3	53	116	30	59.1			
3	24	100	32	55.6	57.3	686	
5	24	200	55	100.7			
5	29	190	45	99.5	100.1	754	721
Cooking water.							
3	31	115	25	70.8			
3	42	138	40	74.1	72.4	888	
5	24	200	60	118.5			
5	41	200	50	122.5	120.5	917	902
Total							1,623
B. Beans cooked at 100°C. in 0.5 per cent $NaHCO_3$ solution.							
None.	15	10	2	6.6			
"	39	13	2	5.2	5.9		
Extract from 30 gm. of beans.							
3	38	108	13	49.6			
3	47	120	21	53.0	51.3	605	
5	27	160	32	95.0			
5	34	160	53	88.8	91.9	688	646
Cooking water.							
3	51	84	10	43.6			
3	61	90	15	49.7	46.6	542	
5	24	112	40	70.4			
5	30	120	25	78.0	74.2	546	545
Total							1,191
Loss, *per cent*							37.5

TABLE IV—*Concluded.*

Quantity added.	No. of cells growing.	Maximum.	Minimum.	Average.	Average of duplicate determinations.	No. of cells for 1 gm. of original material, corrected for blank.	
							Average.
C. Beans cooked at 100°C. Lot II.							
cc.							
None.	20	18	2	6.7	6.7		
Extract from 30 gm. of beans.							
3	23	150	28	86.1			
3	30	160	55	99.4	92.7	1,146	
5	37	320	30	133.4			
5	60	320	26	134.8	134.1	1,019	1,082
Cooking water.							
3	30	140	36	74.0			
3	52	145	18	80.6	77.3	941	
5	27	250	22	125.4			
5	35	270	36	129.3	127.3	964	952
Total							2,034
D. Beans cooked at 15 lbs. pressure for 45 min.							
None.	42	16	2	6.5			
"	18	9	2	5.0	5.7		
Extract from 30 gm. of beans.							
3	68	71	16	33.8			
3	41	67	18	32.8	33.4	368	
5	74	85	10	48.2			
5	36	96	23	47.2	47.5	334	351
Cooking water.							
3	27	118	30	69.8			
3	25	170	20	66.0	67.9	829	
5	21	240	33	117.5			
5	20	208	52	115.1	116.3	884	856
Total							1,207
Loss, *per cent*							40.6

The canned carrots heated at 115°C. for 45 minutes showed no loss of vitamine. The results indicate an apparent increase. This is doubtless due to experimental error. The entire 326 gm. was not used for extracting the juice. A small portion, 74 gm., was weighed out after the carrots had been mashed in the mortar. There was a great deal of juice and it is possible that in removing the sample an excess of the liquid was taken. The results obtained from the one slide with 5 cc. of cooking water give an average growth per gram of fresh vegetable that is 24 per cent higher than the average growth per gram obtained with the 3 cc. quantities. Since the results for the 3 cc. quantities check very closely, they are undoubtedly the more accurate. If only these averages are considered the total growth per gram of fresh material is 561 and for dry weight 4,955.

That there should be no destruction of vitamine under the conditions of this experiment is to be expected. The carrots were packed tightly in the can and there was relatively little water used, about 100 cc. It takes considerable time for heat to penetrate such a mass so that the vegetable could not have been subjected to the high temperature for long. Less of the vitamine was in solution and it may be that the greater acidity of the carrots inhibited the destructive action of autoclaving.

By cooking in 0.5 per cent sodium bicarbonate solution 37.5 per cent of the vitamine of the beans was lost. This concentration is not more alkaline than is frequently used in cooking dried legumes. While the destruction of vitamine is by no means complete it is still worth considering. It is interesting to note that both autoclaving and alkalies seem to have a destructive effect on the vitamine, just as in the treatment of proteins.

An important fact which was also noted by Daniels and McClurg[8] is that so large a proportion of the vitamine is found in the cooking water. This varies, of course, with the amount of water used. In every case except the canned carrots and autoclaved beans it is about half the total. With the beans cooked at 120°C., 70 per cent of the vitamine was extracted in the cooking water, and, with the canned carrots, 36 per cent of the total vitamine was present in the water. This emphasizes again the importance of utilizing the water in which vegetables are cooked.

SUMMARY.

1. The yeast method used for the quantitative determination of the vitamine content of vegetables has yielded good results when averages were taken of a large number of colonies.

2. Cooking carrots at 100°C. for 30 minutes caused no reduction in the vitamine. Neither was there any destruction when carrots were packed tightly in a jar and heated at 115°C. for 45 minutes.

3. Cooking navy beans at 120°C. for 30 minutes decreased the vitamine content 40.6 per cent. In this case the beans were somewhat overcooked.

4. Cooking navy beans in 0.5 per cent sodium bicarbonate solution for 1 hour and 10 minutes caused a loss of 37.5 per cent of the vitamine.

5. A large proportion of the vitamine, from 36 per cent to 70 per cent, was present in the cooking water.

In conclusion I wish to express to Professor F. C. Koch my sincere appreciation of his generosity in giving helpful suggestions and providing equipment, and to Mr. F. K. Swoboda for instruction in the use of the yeast method.

WATER-SOLUBLE B IN CABBAGE AND ONION.

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Through the experiments reported in this paper, it was hoped to determine the effect of cooking on the water-soluble B content of vegetables and to express that effect in a definite quantitative way. Although this latter has not been possible, some significant results have been reached.

Cabbage was used for most of the work, because it is eaten both raw and cooked, and because of its common use among the poor, where it often furnishes one of their best sources of all three vitamines.

Method.

The method used was the yeast growth method as developed by Dr. Roger J. Williams in the Laboratory of Physiological Chemistry of the University of Chicago. In brief, the method consists in examining microscopically hanging drops containing single yeast cells in either a control solution or a solution containing also the substance to be tested for water-soluble B. The number of cells into which the single cells have multiplied in a certain number of hours indicates the presence or absence of the vitamines.

Basal Solution.—The basal solution or control was the same as that used by Miller (1). The control was made up into portions of 25 cc. and these were sterilized for 10 minutes at 15 pounds pressure. They were then kept at room temperature until used.

Sterilization.—The solutions to be tested for water-soluble B were made sterile by the alcohol with which the vitamine had been extracted. That 50 per cent alcohol sterilized the solution was proved in our laboratory since there was no bacterial growth even when the plates were made from a solution which had stood for 3

days. Ordinarily the solutions were tested immediately after being made, but if held over for several days they were kept in the refrigerator.

To secure as nearly sterile conditions as possible, the isolation of the yeast cells, the seeding of the yeast, and the making of the slides were performed in a glass box which was sprayed every few days with alcohol and opened as little as possible to allow work.

Yeast.—The yeast used was the ordinary bakers' yeast (Fleischmann's). It was procured from a local store three times a week and used on 2 days only. Meanwhile it was kept in the refrigerator. It was unwrapped only inside the glass box.

At the time of the experiment, the basal solution, the vitamine solution, and the yeast were taken to the glass box, and the work was performed inside it as follows:

A tiny speck of yeast was removed with a sterile steel pen and suspended in a test-tube containing 10 cc. of sterile water. The suspension was made uniform by blowing into it through a sterile 1 cc. pipette attached to a sterile rubber tube containing a cotton plug. 1 cc. of this yeast suspension was then added by the pipette to the basal solution made up to 30 cc., with sterile water if used as a control, or with the vitamine solution and water to the same volume.

Making of Slides.—As soon as the yeast was added the solution was gently shaken, a sterile pen dipped into it, and 25 very minute drops were made on a cover-slip. These were immediately inverted on hollow slides, and sealed with vaseline. In order to make the drops adhere to the cover-slips, the slips, after being thoroughly cleaned with cleaning solution and distilled water, had been placed in a mixture of benzene and vaseline (1 drop of vaseline to 200 cc. of benzene). They were then wiped with a sterile cloth and dried in the oven before using. Thus a film was left on the slips so that the drops adhered. Two slides at least were made from each solution. The drops were made minute in order that the entire drop might be in the field of vision at one time (with low power), and also to make it more possible to secure drops with only one or two yeast cells. It was impossible to get a suspension in which this was always true but in almost every slide there were at least 8 drops which contained the right number of cells. Not more than six slides were made from one suspension.

Reading of Slides.—When all the slides were made they were examined under the low power of the microscope, and the contents of each drop that contained one or two cells recorded on squared paper, with the relative position of the cells noted, and sometimes the size of the cells also. It was often difficult to recognize cells at this first reading even when the high power was used. After examination the slides were incubated at 30°C. in an electric oven for 18 hours.

They were taken out after 5 or 6 hours and again examined as a check on the first reading. Sometimes what had seemed a single cell at first had grown into such a large colony or such an odd shaped one that it was considered to have grown from a double cell or from the union of two single cells, and was therefore discarded. Sometimes new cells or small colonies were discovered, often near the outer edge of the drop. Usually the single cells had budded or grown to groups of three or four. Occasionally some showed no growth, and sometimes a bit of dirt had been mistaken for a cell. Sometimes the presence of wild yeast was noted at this point.

At the end of 18 hours the final record was taken. Here much judgment was necessary. A single colony or two colonies when their sources could be traced from the beginning gave no difficulty. But when the colonies had broken up into several or changed their position it was hard to feel certain of their source. After seeing two small groups detach themselves from a large one, float off to another part of the drop, and form a new colony normal in appearance, it was felt that not much reliance could be put on the reading in any one drop. Therefore drops showing any unusual appearance were discarded. Sometimes the groups were so large and had grown so much in depth that they could not be counted even under the high power. If they could not be estimated within ten or twenty cells they were discarded. This extremely large growth occurred more often when the single cell in the beginning had been very small, perhaps young, and therefore more vigorous in growth. Drops with wild yeast were of course discarded at this time.

In recording the final results of growth only the growing cells were counted. Williams had recorded also the observed cells which had not grown, but the writer did not feel sufficiently sure

of recognizing cells to warrant doing this. Usually results of duplicate slides were averaged and conclusions as to growth drawn from this joint record.

Comparison with Williams' Method.—The above method is practically the same as that of Williams. The chief differences consist in the method of sterilization. Since the effect of heat on the vitamine solution has been the main point in question, it was necessary to extract and sterilize it without heat. This was accomplished by the use of alcohol. Williams sterilized the mixture of control and vitamine solutions in an autoclave. By making up the control in individual portions, this later sterilization was unnecessary, and the yeast suspension could be added directly to the control and solution.

EXPERIMENTAL.

Controls.—During the entire series of experiments, three different mixtures of basal solution were used. These were tested at various times but a control was not run with each experiment. There were many unaccountable variations in the averages of the controls. In general the first two lots averaged 6 to 8, and the third, 10 to 13. Sometimes duplicate slides averaged exactly the same, but more often there was a difference of 2 to 3. The range of growth in the same slide was often about 3 to 12, but sometimes colonies ran as high as 25. In the tables experiments in which the first and second controls were used are combined since they averaged about the same. The third control was used only in the experiments reported in Tables II, V, VI, and VII.

Cabbage.

Small, firm cabbages were procured at a local store and often used for several experiments extending over 2 or 3 weeks. They were kept in the refrigerator and whenever used a fresh cut was made and a portion cut from that, to avoid the part which had been directly exposed to the air.

Raw Cabbage.—A 10 gm. representative portion of cabbage was used; that is, a vertical section was cut so as to include both outer and inner leaves, the central stalk being discarded. It was cut as fine as possible and ground in a mortar to a soft pulp. 10 cc.

of dilute alcohol (50 per cent) were added in which it stood for 15 minutes. It was then transferred to centrifuge tubes, another 10 cc. portion of alcohol was added, and it was centrifuged for 10 minutes.

The liquid was then decanted off and filtered. Another 10 cc. portion of alcohol was added to the residue in the centrifuge tube, thoroughly mixed, and again centrifuged. This process was repeated four times. The residue was then filtered by suction through a Buchner funnel until dry, and this filtrate added to the previous extractions. Thus the vitamine was extracted with five portions of alcohol. To test the completeness of this extraction the residue on the Buchner funnel was soaked in alcohol, centrifuged twice, filtered by suction, and this extraction tested for water-soluble B. The resulting growth was no larger than the average control showing that no vitamine was present; that is, the extraction was as complete as could be made by this method. Since water-soluble B is more soluble in dilute alcohol than in strong (2), this method should be a fairly complete one.

The vitamine solution was made up to (usually) 100 cc. with alcohol. To a control of 25 cc. was added an amount of vitamine solution equivalent to 0.1 gm. of fresh cabbage and the volume made up to 30 cc. The yeast suspension was then added and slides were made and recorded as explained above.

Table I shows the growth in six experiments on raw cabbage including portions from three cabbages. Many preliminary experiments had been made on the raw cabbage with very similar results. The growth from the 61 cells reported here averages 23.4, with a median of 22 and a range from 11 to 80. The range is large, due to one large colony (80) in Experiment 5. Without that, it would have been 11 to 38. In Experiment 4, the cabbage used had been in the refrigerator for several days and was tough and harder to grind to a pulp, which may have interfered with the extraction of the vitamine and so account for the smaller amount of growth.

Boiled Cabbage.[1]—10 gm. of cabbage, cut as above, were cooked for 30 minutes in 100 cc. of boiling water (distilled) in an uncovered beaker. The boiling was made rapid at the end to evapor-

[1] See preceding paper (Miller, E. W., *J. Biol. Chem.*, 1920, xliv, 159) for review of the literature on stability toward heat and alkali.

ate as much water as possible. What little water remained was decanted off, filtered, and to it was added the extract from the cabbage. This extract was prepared with alcohol similarly to the raw cabbage.

The second part of Table I shows the results of six experiments with boiled cabbage. The growth of 77 cells averages 26.7 with a median of 24 and a range of 7 to 60. The two parts of Experiments 1, 2, and 3 were performed at the same time under as like conditions as possible. In Experiments 1 and 2 there is almost

TABLE I.

Growth from 0.1 Gm. of Cabbage, Raw and Boiled.

Experiment No.	Raw.					Boiled.				
	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median	Average.		Minimum.	Maximum.	Median.	Average.
1	14	13	32	26.5	24.7	12	16	40	25	24.6
2	16	11	29	18	19.4	18	7	37	18	19.8
3	12	11	38	23.5	22.8	13	8	30	17	16.3
4	6	12	26	16	17.3					
5	6	15	80	31	36.2					
6	7	16	33	26	25.1					
7						11	20	48	38	36.8
8						10	7	55	25.5	26.6
9						13	12	60	35	34.4
Total.	61	11	80	22	23.4	77	7	60	24	26.7

exact agreement between the averages of the raw and boiled cabbage. This is the more striking in Experiment 1, for here a second extraction had been made (to test the completeness of the first, as explained above) and a like agreement was shown, an average growth of 9.7 for the raw, and 9.6 for the boiled; that is, approximately the same as the control. Certainly the results indicate that the vitamine is not in the least destroyed by boiling for 30 minutes. Several preliminary experiments had been made on steamed cabbage with similar results.

Table II shows two experiments in which portions were boiled for 30 and 60 minutes, the two parts of the experiment being carried on at the same time. The average growth for the two parts

of the experiments is fairly close, 42.5 for the 30 minute cabbage, and 44.5 for the 60 minute. The averages for the separate experiments, however, are somewhat different. The range in each experiment is also large. (This 30 minute cabbage is not included among the experiments in Table I, because the higher control was used.)

Boiled with Acid.—In two experiments the cabbage was boiled with about 5 cc. of vinegar added to the 100 cc. of water. The resulting growth was very great. The colonies were not only larger than usual but often were broken up into several and were

TABLE II.

Growth from 0.1 Gm. of Cabbage, Boiled 30 and 60 Min.

Experiment No.	30 min.					60 min.				
	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.		Minimum.	Maximum.	Median.	Average.
1	8	12	100	34	40	17	12	140	40	54
2	15	18	100	36	44	12	15	100	34	38
Total.	23	12	100	36	42.5	27	12	140	36	44.5

uncountable. Some new colonies appeared where no cells were recognized on the 5 hour reading. Certainly water-soluble B is not destroyed by boiling with acid. This is in agreement with Williams (3) and with McCollum and Simmonds (4) who find only slight destruction, if any, with nitrous acid or hydrochloric acid.

Boiled with Alkali.—Table III shows the results of three experiments in which the 10 gm. portion of cabbage was boiled in 100 cc. of water to which 0.1 gm. of baking soda was added. The 41 cells show an average growth of 24.1, a median of 21, and a range of 8 to 70. The actual growth, however, was much larger as some broken or uncountable colonies were discarded.

These results with acid and alkali are in agreement with Williams (3) who found that "this material when acted on by acid or alkali is changed (presumably by hydrolysis) to a form which is more immediately available for the yeast."

Loss in Cooking Water.—In this series of six experiments 10 gm. portions of cabbage were boiled in the usual way for 30 minutes. The cooking water, however, was decanted off, mixed with alcohol, and tested separately. The vitamine in the drained cabbage was extracted in the usual way and tested. In testing the cooking water and the cabbage extract amounts equivalent to 0.1 gm. of raw cabbage were used as usual.

TABLE III.

Growth from 0.1 Gm. of Cabbage, Boiled with $NaHCO_3$.

Experiment No.	No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.
1	10	10	70	16	31
2	15	8	42	25	25.1
3	16	9	30	21	18.9
Total.....	41	8	70	21	24.1

TABLE IV.

Growth from Cooking Water and Drained Cabbage.

Experiment No.	Cooking water.					Drained cabbage.				
	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.		Minimum.	Maximum.	Median.	Average.
1	11	6	60	22	28.1	12	10	60	25.5	30
2	16	9	75	24.5	28.4	10	10	70	21.5	28.6
3	9	10	42	20	23.3	15	7	32	14	15.7
4	9	7	36	30	25.4	9	8	24	18	18.1
5	14	16	29	23	22.9	7	15	29	20	19.7
6	3	28	40	29	32.3	8	21	49	29	32.6
Total.	62	6	75	24	26.1	61	7	70	22	23.6

Table IV summarizes the results and shows that in three of the experiments the growth from cooking water and from extract from drained cabbage was practically identical—half the vitamine in each. In the other three, the cooking water contained a trifle more than half. Considering the six experiments together, the 62 cells from the cooking water average 26.1 with a median

of 24, and the cells from the drained cabbage extract average 23.6, with a median of 22. The ranges are very close, 6 to 75 in one case and 7 to 70 in the other.

The combined growth from 0.1 gm. of drained cabbage and its cooking water should equal the growth from 0.1 gm. of boiled cabbage with the water retained (as in Table I). This is nearly true in Experiment 3 in Table IV and Experiment 9 in Table I. These two portions of cabbage were cooked at the same time and the experiments carried out under as like conditions as possible. The control used with these solutions averaged about 8. Allowing for this control the increased growth from the boiled cabbage with the water retained was 34.4 − 8 = 26.4 (Table I, Experiment 9), the growth from the cooking water 23.3 − 8 = 15.3, and from the corresponding extract 15.7 − 8 = 7.7 or a total of 23 from 0.1 gm. of cabbage (Table IV, Experiment 3). These averages, 26.4 and 23, are fairly near.

This series of experiments shows that about half the vitamine is lost in the cooking water. In actual household practice where larger pieces of the vegetable are cooked and therefore less surface is exposed less vitamine would be dissolved out in the cooking water. This retention of more than one-half the vitamine, especially in strongly flavored vegetables such as cabbages and onions (Table VII) where the cooking water is usually discarded, is very important. As far as can be ascertained this is the first attempt to discover the proportion of water-soluble B lost in the cooking water.

Daniels and McClurg (5) warn against discarding water around cooked or canned vegetables because their rats grew on the liquor from cooked beans. But they were dealing with an extraction rather than ordinary "cooking water" since the cooked material was comminuted by pressing through a strainer, the liquor filtered off, and the beans were washed several times with water. In the experiments of Daniels and Byfield (6), in adding water-soluble B to the food of artificially fed babies in order to secure proper growth they used both the alcoholic extract of carrots, turnips, and celery, and a soup made from the same vegetables. Since the water extract was nearly as effective as the alcoholic extract it shows that much of the vitamine had been extracted.

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5	6	15	80	31	36.2					
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	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.		Minimum.	Maximum.	Median.	Average.
1	11	6	60	22	28.1	12	10	60	25.5	30
2	16	9	75	24.5	28.4	10	10	70	21.5	28.6
3	9	10	42	20	23.3	15	7	32	14	15.7
4	9	7	36	30	25.4	9	8	24	18	18.1
5	14	16	29	23	22.9	7	15	29	20	19.7
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Onions.

A few experiments were made on onions. They were cut and treated similarly to the cabbage and the same amounts were used.

Raw Onions.—In two experiments on raw onions (Table V) the average growth from 0.1 gm. of Silver Skins was 35.2, and of Spanish onions 26.1. Of course, this one experiment indicates little as to the relative value of the two kinds but simply shows the presence of water-soluble B in both.

TABLE V.

Growth from 0.1 Gm. of Raw Onion.

Kind.	No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.
Silver Skins.....	20	20	55	35.0	35.2
Spanish.....	16	15	50	25.5	26.1

TABLE VI.

Growth from 0.1 Gm. of Boiled Onion.

Kind.	No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.
Spanish.....	11	11	28	23	21.5
"	4	16	50	41.5	37.2
Total.....	15	11	50	25	25.7

Boiled Onions.—Two experiments on boiled Spanish onions (Table VI) showed an average growth of 25.7, as against 26.1 for the raw Spanish onions. The heating had not destroyed the vitamine.

Loss in Cooking Water.—Two experiments comparing the cooking water with the extract from the boiled onion (Table VII) give the same results as cabbage; namely, that only about one-half the vitamine is lost in the cooking water.

No attempt should be made here to compare the vitamine content of cabbage and of onion. The experiments with onions are too few, and the results in both not sufficiently consistent. How-

ever, the presence of water-soluble B in onions is here shown. This is important in view of the apparent contradiction (7) between the work of Osborne and Mendel (8) who proved its presence by the growth of rats, and of McCarrison (9) whose pigeons, fed on polished rice, butter fat as a source of fat-soluble A, and onions as a source of C, developed polyneuritis even more quickly than those on a diet of polished rice alone. If the antineuritic, antiberiberi vitamine is not identical with the growth-promoting vitamine necessary for rats, as has not yet been definitely proved (10), although generally so considered, these results would show the yeast growth vitamine to be identical with the "growth-promoting" vitamine.

TABLE VII.

Growth from Cooking Water and Drained Onion.

Experiment No.	Cooking water.					Drained onion.				
	No. of growing cells.	Amount of growth.				No. of growing cells.	Amount of growth.			
		Minimum.	Maximum.	Median.	Average.		Minimum.	Maximum.	Median.	Average.
1	22	18	43	35	33.1	14	18	52	35	31.7
2	9	12	50	30	29	15	12	40	25	25.5
Total.	31	12	50	30	31.9	29	12	52	30	28.3

DISCUSSION.

As a strictly quantitative test the method has proved somewhat disappointing. Although duplicate slides and duplicate experiments have sometimes been remarkably consistent in range as well as in average even here the range within the slide has been large. Within the same drop even, 1 cell may grow to 20 and another to 40.

It would seem that the differences in growth of the cells must often be due to a difference in the individual yeast cells themselves. Sometimes they varied in shape and appearance as has been noted. Then, too, there may be some difference in the yeast cakes. Other substances may be introduced with the yeast cells.

The possible errors in reading the slides have already been noted, and most of them have been recognized by Williams.

Among other factors which may have affected the results, in spite of all efforts to standardize the method, may be included: variations in the cabbage—different cabbages, parts of the same cabbage, changes due to keeping; variations in the extract, completeness, time before using; influence of other substances which were probably extracted with the vitamine, such as mineral salts, nitrogenous substances, and carbohydrates; variations in control; and variations in temperature of the glass box which was sometimes much above room temperature.

CONCLUSIONS.

Although strictly quantitative results have not been secured by these experiments certain definite points have been made.

1. Water-soluble B in cabbage is not destroyed by boiling for 30 or 60 minutes.
2. It is not destroyed by boiling with acid or alkali.
3. It is present in onions and is not destroyed by boiling.
4. Not more than one-half the water-soluble B is lost in the cooking water from cabbage or onions boiled for 30 minutes.

I wish to express my thanks to Dr. F. C. Koch, Dr. R. J. Williams, and Miss E. W. Miller for helpful suggestions concerning this method as used in their laboratory.

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CAN "HOME GROWN RATIONS" SUPPLY PROTEINS OF ADEQUATE QUALITY AND QUANTITY FOR HIGH MILK PRODUCTION? II.*

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In 1919[1] we published results which showed that it was impossible to maintain nitrogen equilibrium in dairy cows of high daily milk production with a ration made from a cereal grain, *red clover hay*, and corn silage. The protein content of such a ration was inadequate in quantity or quality for continuous high production without imposing a considerable draft on the cow's protein tissues. We showed that with clover hay as the roughage in the above ration a high milk-producing cow would not only fall off in the volume of milk secreted more rapidly than normally occurs with the progress of lactation, but that she would show a negative nitrogen balance. On the other hand it was shown that it was possible, with cows of moderate milking capacity of 20 to 25 pounds of milk daily containing 14 per cent of solids, but with large feed consumption, to maintain milk flow as well as nitrogen equilibrium over a period of at least 4 months on a ration made of red clover hay, corn silage, and a cereal grain.

We expressed the view that there was a possibility that through further research a combination of "home grown" materials could be found that would be more effective with respect to quantity or quality of proteins than the ration used in our earlier experiments.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1919, xxxviii, 515.

Naturally the very great prominence given to *alfalfa* as a forage plant in all northern areas of this country led us to use it in substitution for red clover hay as a possible material of either greater protein efficiency or higher protein content. The experiments to be described in this paper are based on the use of alfalfa hay, together with corn silage and a cereal grain as the ration. The grains used were corn, barley, oats, or a mixture of the three. As in all work of this kind, metabolism experiments are absolutely necessary if we are to have positive knowledge in regard to the intake and outgo of nitrogen. Without such data no definite information of value on the status of nitrogen metabolism in the animal can be accumulated, especially when short periods of time are involved.

Much to our surprise we found it possible not only to maintain nitrogen equilibrium in cows milking 40 to 45 pounds of 11 per cent milk daily, but to maintain that flow over a period of 16 weeks with a ration made from alfalfa hay, a cereal grain, and corn silage. The alfalfa used was not specially selected, but was taken from a car lot bought by the University as a commercial product and grown in the West. It contained 15.5 per cent of protein (N $\times$ 6.25) which is but 0.6 per cent higher than the average of 250 analyses of this plant as given by Henry and Morrison.[2]

It is true, however, that most of the materials used in the construction of this alfalfa ration were appreciably higher in nitrogen than the materials used in our clover hay ration of the preceding year; but the main difference between the two rations was the higher level of crude protein secured in this ration and made possible by the use of alfalfa. Data showing the differences in nitrogen content of the materials used in the clover ration of 1918–19 and the alfalfa ration of 1919–20 are given in Table I. With the exception of corn silage, the data are based on the air-dried material.

From our metabolism data it does not appear that a unit of nitrogen absorbed from the clover-silage-cereal grain ration was less productive of milk and tissue protein than a unit of nitrogen absorbed from the alfalfa-silage-cereal grain ration. On the

[2] Henry, W. A., and Morrison, F. B., Feeds and feeding, Madison, 15th edition, 1915, 640.

basis of weekly periods there was approximately 0.45 to 0.64 gm. of nitrogen elaborated in product per gm. of absorbed nitrogen on the clover ration, and 0.41 to 0.59 gm. per gm. of absorbed nitrogen on the alfalfa ration. These differences between the effectiveness of the two rations are too slight to be given any significance and should not be accepted as indicating that we had a quality of protein in the alfalfa ration superior to that of the clover ration. The greater efficiency of the alfalfa ration over the clover ration is probably to be attributed to the higher plane of protein intake made possible by the higher nitrogen content of the alfalfa ration. The alfalfa hay itself was mainly responsible for this higher nitrogen level.

TABLE I.

Nitrogen Content of Feeds.

Material.	Nitrogen.	
	1918–19.	1919–20.
	per cent	*per cent*
Corn grain	1.43	1.65
Oat "	1.48	1.70
Barley "	1.80	1.89
Clover hay	1.74	
Alfalfa "		2.48
Corn silage	0.36	0.45

EXPERIMENTAL.

Our plan was to use a ration of corn silage and alfalfa hay to which ground corn was added in the first period of 4 weeks, ground oats in the second period of 4 weeks, ground barley in the third period of 4 weeks, and a mixture of approximately equal parts of the three grains in the fourth period of 4 weeks. The alfalfa hay was cut before feeding. This was done in an ordinary feed cutter. The grains were ground. Provision for a constant energy intake with the different rations was made through the use of corn-starch. In some cases the amount of grain allowed was above, while in other cases it was below, the proportion of 1 pound of grain to 3 pounds of milk—a practical rule often used and recommended by animal husbandry teachers. The animals

were fed all that they would consume but their consumption did not conform closely with the energy needs as set forth in the Armsby standards. For example, Cow 1 consumed 58 pounds of the corn ration which provided 20 therms. On the basis of her milk production (42 pounds of 3 per cent (fat) milk daily) and live weight (1,200 pounds) she should have received 16 therms. Cow 2 consumed 50 pounds of the corn grain ration or its equivalent as dictated by appetite. This provided an intake of 17.2 therms. Her energy requirement as formulated by the Armsby standard based on milk production (30 pounds daily of 3.5 per cent milk) and live weight (1,200 pounds) would have been 14.1 therms.

Our plan had been to use three animals for the four periods of 4 weeks each, but we were not successful in carrying all of them through the entire time. We succeeded in our plan with three of the animals; but No. 3 went off feed at the end of 4 weeks with the necessity of providing a fourth animal for the remaining three periods.

One of the animals used was a pure bred Holstein, the other three were grade Guernseys. They were fed and milked twice daily, with quantitative collection of all excreta. The urine and feces were analyzed daily, while a 7 day composite of the milk was collected for analysis. The composition of the rations fed is shown in Table II. Data are given for 50 pounds of the corn grain ration and its equivalent in protein and therms in the case of the oat, barley, and mixed grain rations. It should be remembered that 50 pounds or its equivalent of any particular ration was not necessarily fed, but that portion allowed which was entirely consumed.

A nutritive ratio of 1:7.9 was made possible with the materials used. The cows differed in their consumption, some eating more than 50 pounds of the ration while others ate less. Cow 1, a Holstein, consumed 58 to 60 pounds of the ration daily, while Cow 3, a smaller animal and producing less milk, consumed 46 to 48 pounds of the ration. In 50 pounds of this ration there were provided 3.44 pounds of *crude* protein. From our records of nitrogen metabolism about 60 per cent of the nitrogen was digested (absorbed). This amounts to a provision of 2.06 pounds of *digestible crude* protein with 50 pounds of the ration. This amount of protein was ample over the period of observation for

Cow 2, producing daily 30 pounds of 3.2 per cent milk (fat) and weighing 1,200 pounds. The Armsby standard would require 1.98 pounds of digestible *true* protein daily for similar performance and with no provision for the influence of the quality of

TABLE II.

Composition of Ration.

	Weight.	Total N.	Therms.	Total protein.
Corn grain ration.				
	lbs.	*gm.*		*lbs.*
Silage	30	62.6	4.96	
Alfalfa	10	112.5	3.44	
Grain	10	74.9	8.88	
Starch				
Total	50	250.0	17.28	3.44
Oat grain ration.				
Silage	30	62.6	4.96	
Alfalfa	10	112.5	3.44	
Grain	9.7	74.9	6.62	
Starch	2.2		2.20	
Total	51.9	250.0	17.22	3.44
Barley grain ration.				
Silage	30	62.6	4.96	
Alfalfa	10	112.5	3.44	
Grain	8.7	74.9	7.02	
Starch	1.8		1.80	
Total	50.5	250.0	17.22	3.44
Mixed grain ration.				
Silage	30	62.6	4.96	
Alfalfa	10	112.5	3.44	
Corn	3.3			
Oats	3.2	74.9	7.52	
Barley	2.9			
Starch	1.3		1.30	
Total	50.7	250.0	17.22	3.44

the protein. On the basis of our analysis of the materials used and the coefficients of digestibility given in the text-books on feeding, 50 pounds of our ration would have provided 2.28 pounds of *digestible crude* protein. This is not in very close agreement with the Armsby standard, if the amount of digestible crude protein supplied by our ration is reduced to *digestible true* protein; in fact it would fall considerably below the provision of the Armsby standard; but there probably can be no question but that the free amino or simple peptide nitrogen of the corn silage and alfalfa hay is of direct nutritive value, although it may not be precipitated by Stutzer's[3] reagent which is used in differentiating crude from true protein. The Wolff-Lehmann standard would require 3.3 pounds of *digestible crude protein* for a 1,000 pound cow, giving 27.5 pounds of milk, while modified standards suggested by Henry and Morrison would require 2.3 pounds of *digestible crude protein* for a 1,000 pound cow giving 30 pounds of 3.5 per cent milk.

In three of the periods extra energy and under practical conditions probably unnecessary energy was provided in the form of corn-starch. This was done to make the oat, barley, and mixed grain rations as high in net energy as the corn grain ration, which, with its corn-meal, contained a higher therm value than the other rations. We set our standard as the corn grain-alfalfa-silage mixture in order to get as much protein into the ration as possible, necessitating thereby an equal therm provision in all the other rations. It can be seen from Table II that we could have omitted from the oat, barley, or mixed grain ration the added energy in corn-starch and still have provided adequate therms and protein.

The records of nitrogen metabolism are shown in Tables III to V. Positive nitrogen balances were maintained with all the animals. The records for Cows 1, 3, and 4 would indicate some protein storage. Cow 2 was nearer nitrogen equilibrium, although the greater part of the time she was in a distinctly positive nitrogen balance. All these animals had been bred in the first 2 weeks of confinement to experimental conditions and part of the nitrogen storage is to be correlated with fetus build-

[3] Stutzer, N., in Hart, E. B., and Bentley, W. H., *J. Biol. Chem.*, 1915, xxii, 477.

ing. The live weights of these animals were well maintained, although no great importance is to be attached to a maintenance of live weight as indicating a positive nitrogen balance, particularly over a period of 3 to 4 months with a cow.

TABLE III.

Record of Nitrogen Balance, Milk Produced, Etc.

Date.	Nitrogen.					Milk per week.
	Intake.	Feces.	Urine.	Milk.	Balance.	
Corn grain ration, Animal 1.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Dec. 5–11, 1919.....	1,910.7	699.0	519.0	554.6	+138.1	305
" 12–18, 1919.....	2,030.7	719.2	515.6	559.8	+236 1	301
" 19–25, 1919.....	2,030.7	824.8	493.2	528.3	+184.4	284
" 26, 1919–Jan. 1, 1920.....	2,030.7	780.4	520.8	576.8	+152.7	291
Oat grain ration, Animal 1.						
1920						
Jan. 2- 8.....	2,025.8	761.5	492.7	582.1	+189.5	290
" 9–15.....	2,025.8	743.2	558.6	568.4	+155.6	288
" 16–22.....	2,025.8	733.0	572.9	538.3	+181.6	285
" 23–29.....	2,025.8	757.6	592.6	559.9	+115.7	293
Barley grain ration, Animal 1.						
Jan. 30–Feb. 5.....	2,025.8	771.6	562.9	568.6	+122.7	303
Feb. 6–12.....	2,025.8	717.5	549.7	562.6	+196.0	292
" 13–19.....	2,025.8	836.6	461.5	582.6	+145.1	288
" 20–26.....	2,025.8	796.9	458.1	561.4	+209.4	290
Mixed grain ration, Animal 1.						
Feb. 27–Mar. 4.....	2,025.8	798.3	429.7	557.9	+239.9	294
Mar. 5–11.....	2,025.8	760.0	465.7	568.3	+231.8	294
" 12–18.....	2,025.8	782.9	457.5	559.8	+225.6	287
" 19–25.....	2,025.8	800.4	480.7	568.2	+176.5	290

The initial and final live weights of these animals were as follows: No. 1, 1,174 to 1,250; No. 2, 1,220 to 1,188; No. 3, 1,017 to 1,007; No. 4, 1,117 to 1,108 pounds.

The milk production, both in composition and flow, was well maintained. It is exactly on this point that our data cannot give a final answer to the question whether the milk flow and milk composition would have been maintained over an entire

TABLE IV.

Record of Nitrogen Balance, Milk Produced, Etc.

Date.	Nitrogen.					Milk per week.
	Intake.	Feces.	Urine.	Milk.	Balance.	
Corn grain ration, Animal 2.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Dec. 5–11, 1919.....	1,751.4	573.0	607.4	461.1	+109.9	246
" 12–18, 1919.....	1,751.4	641.6	677.7	445.8	−13.7	242
" 19–25, 1919.....	1,751.4	620.0	586.5	437.1	+107.8	220
" 26, 1919–Jan. 1, 1920.....	1,751.4	611.0	705.9	442.8	−8.3	220
Oat grain ration, Animal 2.						
1920						
Jan. 2–8.....	1,750.0	619.2	626.2	436.2	+68.4	215
" 9–15.....	1,750.0	645.7	648.1	433.3	+22.9	215
" 16–22.....	1,750.0	577.0	714.9	402.3	+55.8	196
" 23–29.....	1,750.0	752.2	630.8	443.9	−76.9	211
Barley grain ration, Animal 2.						
Jan. 30–Feb. 5.....	1,750.0	676.5	597.9	433.4	+42.2	219
Feb. 6–12.....	1,750.0	621.8	583.0	417.0	+128.2	209
" 13–19.....	1,750.0	677.0	645.2	416.1	+11.7	205
" 20–26.....	1,750.0	622.3	551.1	423.6	+153.0	197
Mixed grain ration, Animal 2.						
Feb. 27–Mar. 4.....	1,750.0	647.8	564.6	416.8	+120.8	204
Mar. 5–11.....	1,750.0	628.8	598.7	422.7	+99.8	211
" 12–18.....	1,750.0	680.4	620.9	420.1	+28.6	201
" 19–25.....	1,750.0	674.9	632.7	419.9	+22.5	212

gestation period by a ration providing no more protein than the one we used. We can only answer the question for a period of 4 months. Over that time the volume of milk secreted, as well as its composition, was well maintained. Only that shrinkage

in volume incident to a progressive lactation was observed. The composition of the milk and average daily production for a single week in each feeding period are given in Table VI. Charts 1 to 3 are added to show the maintenance of nitrogen equilibrium as

TABLE V.

Record of Nitrogen Balance, Milk Production, Etc.

Date.	Nitrogen.					Milk per week.
	Intake.	Feces.	Urine.	Milk.	Balance.	
Corn grain ration, Animal 3.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Dec. 5–11, 1919.....	1,610.7	612.1	479.6	435.8	+83.2	178
" 12–18, 1919.....	1,610.7	566.4	444.4	458.2	+141.7	180
" 19–25, 1919.....	1,610.7	627.1	397.3	448.0	+138.3	170
" 26, 1919–Jan. 1, 1920.....	Off feed.					
Oat grain ration, Animal 4.						
1920						
Jan. 2– 8.....	1,610.0	605.4	488.5	501.5	+14.6	217
" 9–15.....	1,610.0	504.0	462.4	507.7	+135.9	217
" 16–22.....	1,610.0	588.3	489.2	463.3	+69.2	195
" 23–29.....	1,610.0	591.1	485.4	484.0	+49.5	212
Barley grain ration, Animal 4.						
Jan. 30–Feb. 5.....	1,610.0	606.3	503.3	493.5	+6.9	211
Feb. 6–12.....	1,610.0	528.6	502.2	481.1	+98.1	201
" 13–19.....	1,610.0	569.7	453.7	465.5	+121.1	188
" 20–26.....	1,610.0	551.7	461.3	459.6	+137.4	188
Mixed grain ration, Animal 4.						
Feb. 27–Mar. 4.....	1,610.0	563.0	444.0	447.4	+155.6	189
Mar. 5–11.....	1,610.0	636.4	420.2	460.2	+93.2	194
" 12–18.....	1,610.0	640.2	439.7	458.7	+71.4	194
" 19–26.....	1,610.0	630.1	450.2	455.9	+73.8	192

well as the constancy in milk nitrogen elaboration over the 16 weeks of observation.

In our earlier paper reviewing our experience with clover hay we stated that "from what we know of the physiology of milk

secretion and the relation of protein to that function it would appear to be unprofitable not to supply protein in quantities sufficient to maintain nitrogen equilibrium in a milking animal." We believe that to be an axiom of good dairying, but we must also insist that the set nutritive ratio for milk production is as fallacious, scientifically, as the set nutritive ratio for growth. In the light of modern protein chemistry our knowledge of the protein requirements of dairy cows is in as confused a state as our knowledge of the protein requirements for human nutrition.

TABLE VI.

Composition of Milk and Average Daily Flow in a Selected Week of Each Period.

	Animal 1.			
	Dec. 18.	Jan. 15.	Feb. 12.	Mar. 11.
Total solids, *per cent*	11.40	11.50	11.40	11.30
Fat, *per cent*	3.00	3.00	3.00	3.00
Nitrogen, *per cent*	0.41	0.43	0.42	0.43
Milk daily, *lbs*	43.00	41.10	41.70	42.00
	Animal 2.			
Total solids, *per cent*	11.40	11.40	11.90	11.70
Fat, *per cent*	3.20	3.20	3.60	3.50
Nitrogen, *per cent*	0.40	0.40	0.43	0.44
Milk daily, *lbs*	34.50	30.70	29.80	30.10
	Animal 4.			
Total solids, *per cent*		13.40	13.65	14.00
Fat, *per cent*		4.40	4.80	5.00
Nitrogen, *per cent*		0.51	0.51	0.52
Milk daily, *lbs*		30.00	28.70	27.70

There are advocates of high levels as well as advocates of more moderate levels. The guiding facts will only come to light when quality and quantity of protein are involved in experimental inquiry, through the use of metabolism experiments. In the present state of our knowledge a nutritive ratio set at a high level becomes safe practically, and that of course justifies its use until we have positive evidence that high levels of protein are injurious or unnecessary with certain specific mixtures.

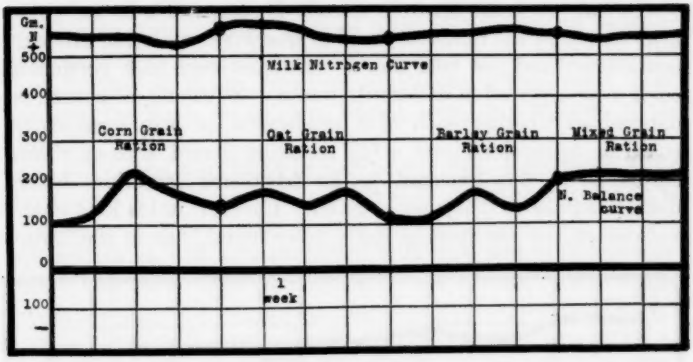

CHART 1. Animal 1, showing nitrogen balance and milk nitrogen produced. A positive nitrogen balance as well as a constant amount of milk ntrogen was maintained for 16 weeks.

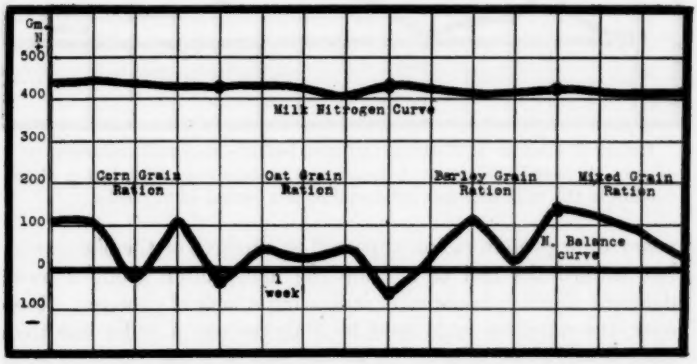

CHART 2. Animal 2, showing nitrogen balance and milk nitrogen produced. Nitrogen equilibrium as well as a constant amount of milk nitrogen was maintained for 16 weeks.

In the experiments reported here it is evident that we have in a cereal grain-corn silage-*alfalfa* ration a "specific mixture" which can, when the nitrogen content is comparable to the nitrogen content of the materials used in this work, maintain nitrogen equilibrium and the milk flow of comparatively high producing cows over a period of at least 16 weeks. It should, of course, be determined whether such milk production as here experienced could be maintained over a full lactation period, with only that shrinkage in flow incident to an advancing lactation, before definite advice to dairymen is given. Haecker[4] in his long observations on the nutrition of dairy cows found that in the earlier

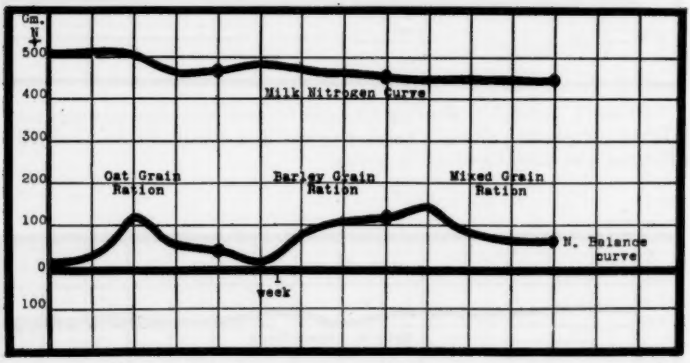

CHART 3. Animal 4, showing nitrogen balance and milk nitrogen produced. A positive nitrogen balance was maintained, with but a slight decline in the milk nitrogen produced over a period of 12 weeks.

years a low protein ration appeared as effective as the higher one, but toward the end of 3 years the low protein group showed deficient vitality, apparently indicating a lack of protein. However, the nutritive ratio used by Haecker was a wider one than used in our experiments. It should also be made perfectly clear that our ration involved the use of a cereal grain in addition to corn silage and alfalfa and was not made up from the two latter materials alone. Further, we could observe no differences in the nutritive efficiency of our rations whether the grains used were corn, oats, barley, or a mixture of the three.

[4] Haecker, T. L., *Minnesota Exp. Station, Bull. 71*, 1901; *Bull. 79*, 1903; *Bull. 140*, 1914.

SUMMARY.

1. In this paper are presented data which show that it is possible to furnish dairy cows of high milk-producing capacity with a protein level of sufficient magnitude to maintain that capacity for 16 weeks from an alfalfa-corn silage-cereal grain mixture, the latter being corn, barley, oats, or a mixture of the three.

2. Four cows were used in this work. No. 1 produced 40 to 45 pounds of 11.4 per cent (solids) milk daily over a period of 16 weeks, on this "home grown" ration, maintaining nitrogen equilibrium as well as a sustained milk flow and milk composition. The other three animals were not so large producers as No. 1, but with less ration maintained a positive nitrogen balance and sustained milk flow and milk composition over the period of observation.

3. This ration had a nutritive ratio of 1:7.9 and provided less *digestible true* protein than the requirements of the Armsby standard. It provided somewhat less *digestible crude* protein than required by the modified standards suggested by Henry and Morrison. Longer observations must decide whether such a ration is adequate for an entire lactation period or for the practical period of barn feeding, unsupplemented by pasture grasses.

NOTE ON THE PRESERVATION OF SPECIMENS OF BLOOD INTENDED FOR BLOOD SUGAR DETERMINATIONS.

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(Received for publication, July 16, 1920.)

The relatively rapid decrease in the sugar content of blood, even when preserved at low temperatures, is too well known to require comment.

Both from the standpoint of the biochemist and of the clinical worker there is great need of some simple method whereby samples of blood intended for sugar determinations might be preserved, if necessary for several days, until some suitable time or place may be found in which the analyses can be made. This need is particularly acute in hospital work, and we have, therefore, experimented with a number of substances, which would be expected, theoretically at least, to limit enzyme action and at the same time to introduce no errors in the subsequent determination of blood sugar by the revised method of Folin and Wu,[1] the procedure used as a routine in this laboratory.

We believe that we have found a suitable preservative in formaldehyde, which we use in the proportion of 1 drop of commercial "formalin" (containing 40 per cent formaldehyde) to 5 cc. of oxalated blood. The dropper employed gives 30 drops per cubic centimeter. The addition of a larger amount of formaldehyde (3 drops to 5 cc. of blood) adds no advantage, nor apparently does it introduce any error.

We are aware of the fact that formaldehyde is ordinarily classed as a weak reducing agent, and that the advisability of its use in this connection may be questioned on theoretical grounds. In fact, we only tried it after we had exhausted every other possibility. We have, however, repeatedly demonstrated the fact

[1] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

that formaldehyde in amounts ten times greater than used in the blood sugar determinations produces no formation of cuprous oxide in the alkaline copper tartrate solution used in the Folin-Wu procedure, a fact probably due to the extremely low degree of alkalinity possessed by this reagent.

The following experimental data is cited in proof of the above statements.

Blood No. 12.

Amount of blood sugar per 100 cc.	Amount of formaldehyde.
mg.	*drops*
99.	None.
100.	1
100.	2
100.	3

Blood No. 16.

Amount of blood sugar per 100 cc.	Amount of formaldehyde.
mg.	*drops*
101.	None.
102.	1
101.	2
101.	3

It will be found that if to 2 cc. of the tungstic acid filtrate there is added 1 drop of formaldehyde, and the sugar determination is made on this portion of filtrate in the usual way, results will be secured which are identical with those obtained in portions of the same filtrate to which no formaldehyde has been added.

By means of formaldehyde used in the manner above described we have been able to prevent glycolysis in blood for as long as 96 hours at temperatures varying from 20–33°C. It seems probable that this period might be greatly extended, but as 4 days appeared to cover all ordinary needs we have not extended our experiments to cover a longer period of time.

We have also made a few observations to determine whether the formaldehyde-containing filtrate can be used for the determinations of the various non-protein blood constituents, such as non-protein nitrogen, urea, creatinine, and uric acid. While

TABLE I.

Experimental Results Showing the Effect of Formaldehyde in Preventing Glycolysis in Non-Diabetic Blood.

Sample No.	Amount of sugar per 100 cc. of blood.			
	20 to 60 min. after bleeding.	After 24 hrs.	After 36 hrs.	After 72 hrs.
	mg.	*mg.*	*mg.*	*mg.*
2	98	100		
3	103	107		
4	91	92		
1	95		95	
5	100		100	
6	90		94	
12	99			100
9	138			138
13	116			119
17	82			82
15	100			100

TABLE II.

Experimental Results Showing the Effect of Formaldehyde in Preventing Glycolysis in Diabetic Blood.

Sample No.	Amount of sugar per 100 cc. of blood.		
	20 to 60 min. after bleeding.	After 48 hrs.	After 96 hrs.
	mg.	*mg.*	*mg.*
22	210		214
23	125	125	
24	158	161	
25	156	162	
26	128	121	133
27	196	186	200
29	232	232	232
30	188	184	190
31	146	143	150
32	143	145	
33	174	178	
34	154	160	
35	202	208	
37	121	122	

we have been able to obtain good results in the determination of these two latter constituents, the values obtained for non-protein nitrogen and for urea (determined by the urease method) are invariably low.

The results presented in Tables I and II indicate the possibilities of this method of preservation. The samples of non-diabetic blood were taken in the afternoon soon after the midday meal; all the samples of diabetic blood were obtained from fasting subjects.[2]

[2] For the diabetic material we are indebted to Dr. Elliott P. Joslin.